No. 22-16770

IN THE UNITED STATES COURT OF APPEALS
FOR THE NINTH CIRCUIT

NATURAL GROCERS, *et al.*,

Plaintiffs-Appellants,

v.

THOMAS J. VILSACK, et al.,

Defendants-Appellees,

and

UNITED STATES BEET SUGAR ASSOCIATION, THE AMERICAN SUGARBEET GROWERS ASSOCIATION, AND THE AMERICAN FARM BUREAU FEDERATION,

Intervenor-Defendant Appellees.

On Appeal from the United States District Court, Northern District of California
Case No. 20-5151 (Hon. Judge James Donato)

INTERVENOR-APPELLEES UNITED STATES BEET SUGAR ASSOCIATION, THE AMERICAN SUGARBEET GROWERS ASSOCIATION, AND THE AMERICAN FARM BUREAU FEDERATION EXCERPTS OF RECORD INDEX VOLUME

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March 8, 2024

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SUPPLEMENTAL EXCERPTS OF RECORD VOLUME 1 of 2

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March 8, 2024

United States Beet Sugar Industry

August 25, 2017

Mr. Bruce Summers
Acting Administrator
Agricultural Marketing Service
United States Department of Agriculture
1400 Independence Avenue, SW
Room 3069 South Building
Washington, DC 20250

Submitted via GMOlabeling@ams.usda.gov

Re: Stakeholder Input on Questions Regarding the Establishment of a National Bioengineered Food Disclosure Standard.

Dear Mr. Summers:

This submission is made on behalf of the United States Beet Sugar Industry representing all of the 10,000 progressive family farmers of sugarbeets in 11 states, who own all nine farmer cooperatives (22 factories), the cooperatives employees, seed producers and the scientists that are engaged in the production and processing of sugarbeets. We produce 56% of the sugar grown in the U.S. We raise sugarbeets on 1.2 million acres, provide 100,000 jobs and generate $10.6 billion for the U.S. economy. We proudly provide the highest quality of sugar for both the safety of our food supply and the food security of our nation. The sugarbeet is one of the best suited plants for use in biotechnology and we have produced 100% bioengineered plants since 2015.

We appreciate the opportunity to share our views and perspectives in response to the USDA Agricultural Marketing Service's ("AMS") request to address outstanding issues or clarifications AMS is considering in preparing a proposed rule to implement the National Bioengineered Food Disclosure Standard, Pub. L. 114-216, (the "Act" or "Disclosure Standard"). Because the Disclosure Standard was not enacted to address the safety, health, or nutrition of bioengineered crops or ingredients, the beet sugar industry's principal concern is that AMS not in any way cause the market to discriminate against biotechnology. For over 25 years activists, and to some extent farmers using competing production methods, have attacked and maligned biotechnology directly or indirectly in order to grow market share and drive biotechnology out of the food production system. For these reasons, we focus our comments largely on the scope of the Disclosure Standard and its focus on foods containing or not containing bioengineered genetic material. As explained in detail below, sugar produced from sugarbeets bioengineered to be resistant to the herbicide glyphosate is molecularly identical to sugar produced from conventional sugarbeets and from conventional and organic sugarcane. AMS therefore should not alter the definition of a bioengineered food under the Act or establish a threshold that would negatively differentiate beet sugar from all other sugar when there is no legal or scientific basis

to do so. Rather, AMS should, as Congress intended, determine that refined food products that can substantiate the absence of genetic material in the food, are not considered bioengineered under the Act.[1]

The American farmer is an innovator and is committed to growing healthy food for an expanding hungry world in a safe and sustainable manner. America is a global leader in biotechnology and the world will look to AMS as it fashions the regulations to ensure that the technology has a strong foundation for the future, while it informs consumers of its safety and presence in the food supply. It is a time to lead on the science and not acquiesce to unfounded fears.

We appreciate your thoughtful consideration of our submission and stand ready, along with counsel, to answer further questions or supplement additional details should you request them.

Respectively submitted,

American Sugarbeet Growers Association

U.S. Beet Sugar Association

Big Horn Basin Beet Growers Association

Big Horn County Sugar Beet Growers Association

California Beet Growers Association, Ltd.

Colorado Sugarbeet Growers Association

Elwyhee Beet Growers Association

Idaho Sugar Beet Growers Association

Michigan Sugar Company

Minn-Dak Farmers Cooperative

Montana-Dakota Beet Growers Association

Nebco Beet Growers Association

Nebraska Sugar Beet Growers Association

Nyssa-Nampa Sugarbeet Growers Association

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[1] Report of the Committee on Agriculture, Nutrition, and Forestry on S. 2609, December 9, 2016 at 3, (hereinafter "Legislative History")("Congress intends the Secretary to provide exemptions and other determinations under which a food is not considered bioengineered.").

Red River Valley Sugarbeet Growers Association

Southern Minnesota Sugar Cooperative

Southern Montana Sugarbeet Growers Association

Wyoming Sugar Company, LLC

Beet Sugar Development Foundation

American Society of Sugar Beet Technologists

Sugar Industry Biotech Council

U.S. Beet Sugar Industry Comments

The U.S. Beet Sugar Industry provides comments on Questions 1, 4, 8, 9, 10, 12, 23, and 30.

QUESTION 1

What terms should AMS consider interchangeable with 'bioengineering'? (Sec. 291(1))

Context: *The disclosure standard would be a mechanism to inform consumers about their food. AMS is considering the advantages and disadvantages of allowing the use of other terms to provide for disclosure.*

AMS should not use terms other than "bioengineering" because alternative terms will lead to confusion and misinterpretation of the scope of the disclosure standard, which would be directly contrary to Congress's intent to bring clarity and uniformity to the marketplace. Congress gave the term "bioengineering" a precise meaning from which the regulations should not deviate.

We recognize that food manufacturers whose products are not subject to the Disclosure Standard may nevertheless voluntarily disclose information about ingredients in the food. In the interest of uniformity, we urge AMS to provide guidance to manufacturers on appropriate terminology to use and make clear that any voluntary terminology used is not interchangeable with the statutory and regulatory definition of "bioengineering." For example, the terms "genetic engineering" or "Genetically Modified Organism" or "GMO" are inconsistent with Act. Congress intentionally used the term "genetic engineering," rather than "bioengineering" in the preemption provision (Section 295) to broadly preempt state, tribal, and local requirements regarding genetically engineered foods "regardless of whether the technology used to develop the food or seed falls within the definition of bioengineering."[2] Thus, Congress clearly viewed genetic engineering and bioengineering as different – not interchangeable – terms. The terms "Genetically Modified Organism" or "GMO" incorrectly imply that the food contains an "organism," when most foods do not contain organisms. The term "modification" also encompasses a broader range of technologies than in vitro recombinant deoxyribonucleic (DNA) techniques to which the Disclosure Standard is limited. In addition, terms non-genetically modified organisms or Non-GMO have been and are currently being used on food packaging to suggest to consumers that Non-GMO foods are healthier or safer than bioengineered foods, directly contradicting science and FDA's determination that approved bioengineered foods carry no more risk than conventional or organic food. Here, Congress was clear that the Disclosure Standard must not disparage biotechnology and thus the terms "Genetically Modified Organism" or "GMO" should never be confused with the term "bioengineering."

[2] Legislative History at 6.

QUESTION 4

Will AMS require disclosure for food that contains highly refined products, such as oils or sugars derived from bioengineered crops? (Sec. 291(1)(A))

Context: *Many processed foods may contain ingredients derived from bioengineered crops, such as highly refined oils or sugars that contain undetectable levels of bioengineered genetic material such that they are indistinguishable from their non-engineered counterparts. AMS is considering whether to require disclosure for foods containing those derived ingredients that may be undetectable as bioengineered.*

USDA is incorrectly using the term "highly refined ingredients" to refer to food products such as sugar. Rather, the more appropriate term is simply "refined ingredients." Highly processed or refined ingredients typically refer to multi-ingredient mixtures processed to the extent that they are no longer recognizable as their original plant/animal source, e.g., candy, tomato sauce, ice cream, etc. In contrast, when a single isolated food component, such as sugar, is obtained by extraction or purification using physical or chemical processes, it is typically referred to as "refined."[3] For these reasons, we urge USDA to use the term "refined ingredients" when referring to single food components such as sugar.

Requiring disclosure for foods containing undetectable levels of genetic material would contravene Congressional intent and would exceed AMS's authority

The Disclosure Standard is ***unambiguous;*** Congress required disclosure only for foods that ***contain bioengineered genetic materials***. Congress thoughtfully, deliberately and intentionally did not extend the scope of the Act to include crops derived from bioengineered plants. Congress further directed the Secretary to "determine the amounts of a bioengineered substance that may be present in food, as appropriate, in order for that food to be a bioengineered food." § 293(b)(2)(B). Thus, any food that does not contain the level of genetic material the Secretary determines to be appropriate for being considered a bioengineered food, cannot be considered a bioengineered food. The Act's legislative history reinforces the plain language of the statute:

> "The Secretary of Agriculture is directed to establish a mandatory uniform national disclosure standard for human food that is or may be bioengineered. For this purpose, *the definition of bioengineering is set in statute and establishes the scope of the disclosure standard.* Congress intends an item of food to be subject to the definition if it contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and this same

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[3] *See e.g.,* Poti, J.M., et al., Is the degree of food processing and convenience linked with the quality of food purchased by US households?, 101 *Am. J. Clin. Nutr.* 1251-1262 (June 2015). *See also*, Monteiro, CA, et al., A new classification of foods based on the extent and purpose of their processing, 11 *Cad Saude Publica*, 2039049 (Nov. 2010) (describing three categories of processed foods: (1) minimally processed foods (physical processes applied to single basic foods such as cleaning, chilling, etc.; (2) processed foods (extraction of one specific component of a single basic food, such as oils and fats, sugar, high fructose corn syrup, and milk and soy proteins); and (3) ultra-processed foods (processing of several foodstuffs, including ingredients from group 2 and unprocessed or minimally processed basic foods from group 1).

> modification could not be otherwise obtained through conventional plant breeding or found in nature."[4]

Refined food products that do not contain genetic material ***do not*** meet the statutory definition of a bioengineered food.

Some groups may argue that Congress defined "bioengineering" in § 291(1) of the Act and gave the Secretary discretion in § 293(a) to define a bioengineered food. They say this reading of the Act is consistent with floor statements made by Members during debate and with a memo from USDA's General Counsel, which some incorrectly describe as a legal opinion. We believe that these groups are reading Member statements and the memo out of context. Nevertheless, they cannot supplant the plain language of the Act. As the Supreme Court has repeatedly made clear the "plain language" of a statute is the "'primary guide'" to Congress' preferred policy." *Sandoz, Inc. v. Amgen, Inc.*, 137 S. Ct. 1664, 1678 (2017) (quoting *McFarland v. Scott*, 512 U.S. 849, 865 (1994). Here, the plain language makes clear that "bioengineering . . . *with respect to a food, refers to a food* . . . that *contains genetic material.*" § 291(1). It further directs the Secretary to set the threshold above which a food is considered a bioengineered food. § 293(a)(2)(B). There is no provision in the Act where Congress gave the Secretary the discretion to rewrite the definition of a bioengineered food from a food that itself contains genetic material to any food derived from bioengineering, a definition Congress expressly rejected. We urge AMS to reject all attempts to broaden the definition of a bioengineered food.

AMS should not assume that a refined food product that does not contain "detectable" amounts of bioengineered genetic material may nevertheless contain bioengineered genetic material and therefore is subject to the Disclosure Standard

Assuming that a refined food product that does not contain "detectable" amounts of genetic material may nevertheless contain genetic material and therefore should be subject to the Disclosure Standard is not scientifically supportable, inconsistent with the Act, at odds with international precedents, and is false and misleading. Also, in the case of sugarbeets, it contravenes scientific evidence that glyphosate tolerance can be achieved through conventional breeding techniques.

1. ***Assuming that a refined food product like beet sugar that does not contain "detectable" amounts of genetic material may nevertheless contain genetic material and therefore should be subject to the Disclosure standard is not scientifically supportable***

Sugar is the case in point: At the molecular level all refined sugar is the same regardless of the plant's genetic makeup (beet or cane) or the production method (Bioengineered, Conventional or Organic) in which the crop was produced. All the genetic material is removed during processing.

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[4] Legislative History at 3.

a. Peer-reviewed scientific studies establish that all genetic material is removed during sugar processing[5]

In 1998, seven years before glyphosate resistant sugarbeets were deregulated in the U.S. and 10 years before their major cultivation in the U.S., German scientists with the Institute of Industrial Genetics at the University of Stuttgart published a study on the fate of DNA and protein during the standard purification steps of the sugar extraction process from both conventional sugarbeets and sugarbeets genetically engineered with the coat protein CP21 to confer resistance to a certain virus.[6] Sugarbeet plant DNA was present in the raw juice from conventional sugarbeets, but was rapidly degraded and removed in the clarification process. In fact, the researchers estimated that the clarification process had the potential to reduce the amount of sugarbeet DNA by a factor of ten to the fourteen (a hundred trillion), which exceeds the total amount of DNA present in sugarbeets. The coat protein CP21 was similarly found in the raw juice from the transgenic sugarbeets, but it too was removed in the clarification process. It was not found in the pulp, thin juice, thick juice, or sugar produced from the transgenic sugarbeets. The researchers therefore concluded that sugar produced from conventional and transgenic sugarbeets is indistinguishable.

Japanese researchers conducted a similar study that also found that sugarbeet plant DNA is degraded and removed in the early stages of the sugar extraction process and is therefore not present in the finished sugar.[7]

b. Industry studies further confirm that beet sugar contains no genetic material

Initially, as part of the deregulation protocol in the USDA/EPA/FDA Coordinated Framework for Regulation of Biotechnology, sugar from transgenic sugarbeets extracted in a laboratory was submitted to the FDA by the technology provider, showing that no transgenic protein or DNA was present.[8] Data submitted in support

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[5] Sugar is extracted from the root of the beet in a multistep process. Sugarbeets are first washed and sliced into thin strips and then placed into a diffuser tank where raw beet sugar juice is extracted with hot water. The raw juice is then "clarified" using excess calcium hydroxide and lime water called milk of lime and carbonation, where carbon dioxide is bubbled through the mixture to form calcium carbonate. Non-sugar particles including genetic material attach themselves to the calcium carbonate and settle to the bottom of the clarifying tanks. The juice is then filtered, resulting in a golden light brown clarified thin juice. At this point, there is no genetic material in the sugar. The thin juice is then boiled and concentrated through the removal of water to form a thicker juice and eventually sugar crystals. The resulting mix of sugar crystals and molasses-rich syrup is then sent to centrifuges for separation. The molasses syrup is spun off and the white sugar crystals are removed.

[6] Klein, J., Altenbuchner, J., and Mattes, R., Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugarbeets. *J. of Biotechnology*, 60: 145-153 (1998). *See* Attachment 1.

[7] Oguchi, T., et al., Investigation of residual DNAs in Sugar from Sugar beet (*Beta vulgaruis L.*), *J. Food Hyg. Soc. Japan*, 50: 41-46 (2009), *available at* https://www.jstage.jst.go.jp/article/shokueishi/50/1/50_1_41/_pdf.

[8] *See* FDA Biotechnology Notification of Food No. 90.

of the consultation also demonstrated that the concentrations of the CP4-EPSPS protein in the roots of the sugarbeet are very low (mean of 161 μg/g).[9] The very low level of the CP4-EPSPS protein in the roots, as well as the transgenic DNA in the sugarbeet tissue, are removed in the sugar extraction process.

Prior to commercial planting and sale of refined sugar into the commercial market, owners of the beet sugar farmer-owned cooperatives sought to reassure food manufacturers and individual customers that beet sugar produced from bioengineered sugarbeets was no different than conventional beet, cane, or organic refined sugar. Thus, in 2006 the Beet Sugar Development Foundation coordinated two studies to confirm the absence of transgenic DNA and the CP4-EPSPS protein in sugar produced from transgenic sugarbeets.

In the first study an independent, internationally respected analytics firm collected samples from each stage of the refining process (three samples each at the start, middle, and end of raw sugarbeet slicing to the finished sugar) at one processing facility. Using methods validated by the European Commission Joint Research Center,[10] the study demonstrated that while transgenic DNA and the CP4-EPSPS protein was detected in the raw sugarbeet and the raw juice, it was not detected at any other subsequent point in the refining process. Thus, consistent with the German study, the study confirmed that the transgenic DNA and CP4-EPSPS protein are removed early in the process at the clarification stage during the transformation from raw juice to thin juice.

In the second study, multiple samples of sugar produced from transgenic and conventional sugarbeets and sugarcane from around the world were analyzed for the presence of plant (plastid) DNA. More specifically, the study sampled organic sugar from Europe, South America and the U.S.; turbinado/muscovado sugar from Africa, Mauritius, and the U.S.; white beet sugar from Canada, Europe, and the U.S. (including sugar produced from transgenic sugarbeets); and white cane sugar from Africa, Australia, Canada, the Caribbean, Europe, Japan, and the U.S.[11] No plant DNA was detected in any of the samples, thus again confirming the German findings that the clarification process effectively removes *all* plant DNA (by a factor of 10^{14}).

In 2014, the Beet Sugar Development Foundation conducted a third study of all U.S. beet sugar factories. Sixty-nine samples of refined sugar were collected from all North American beet sugar factories (three random samples from each of the 22 U.S. factories and the one and only Canadian factory) by the same independent analytic

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[9] Only the roots of the sugarbeet are used in the production of sugar.

[10] Mazzara M., Foti N., Savini C., Van Den Eede G.; "***Event-Specific Method for the Quantitation of Sugarbeet Line H7-1 Using Real-Time PCR - Validation Report and Protocol,***" Online Publication (2006); http://gmo-crl.jrc.ec.europa.eu/gmomethods/entry?db=gmometh&id=qt-eve-bv-001&rq=id%3aQT-eve-BV*.

[11] Forty-four samples of sugar were analyzed, as well as four samples of laboratory pure (analytical grade) sucrose.

firm to test for any presence of transgenic DNA and the CP4-EPSPS protein. A polymerase chain reaction (PCR) test specific for the detection of trace amounts of DNA from the transgenic sugarbeet was used. **All 69 samples of commercial sugar tested negative for transgenic sugarbeet DNA**. All samples were further analyzed for the presence of the particular novel protein, CP4-EPSPS, which confers Roundup® tolerance to the H7-1 Roundup Ready® sugarbeet plant. A commercially available protein test kit for CP4-EPSPS (Romer, Union, MO #7000014) was used for this analysis**. None of the sixty-nine samples showed any detectable CP4-EPSPS protein.** This comprehensive study reaffirmed the 2006 study and the scientific literature that shows that there is no transgenic DNA or protein in the sugar extracted from transgenic sugarbeets.[12]

In sum, the science demonstrates that the sugar extraction process removes all plant DNA regardless of whether the plant is conventional, organic, or transgenic. It would be erroneous for AMS to assume otherwise.

2. Assuming that a refined food product that does not contain "detectable" amounts of genetic material may nevertheless contain genetic material and therefore should be subject to the Disclosure Standard is inconsistent with the Act

Assuming that even if a refined food product does not contain "detectable" amounts of bioengineered genetic material, it may nevertheless contain bioengineered genetic material and therefore should be subject to the Disclosure Standard would render superfluous Congress's direction that the Secretary "determine the *amounts* of a bioengineered substance" that may be present in food to be considered a bioengineered food because AMS is not specifying a threshold. Rather, AMS would be incorrectly assuming that any food derived from bioengineering must contain bioengineered genetic material even if the material cannot be detected through validated scientific methods.

In the case of refined sugar, the science unequivocally demonstrates that the sugar refining process reduces the amount of sugarbeet DNA by a factor of ten to the fourteen (a hundred trillion), which exceeds the total amount of DNA present in sugarbeets. Thus, refined sugar does not contain any plant DNA or proteins, transgenic or otherwise. Should AMS assume that beet sugar contains genetic material for purpose of the Disclosure Standard it would be rewriting the statutory definition of a bioengineered food and arbitrarily mandating disclosure.

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[12] Since highly specific, state-of-the-art tests do not detect any transgenic DNA or protein, both the sugar and molasses extracted from glyphosate tolerant sugarbeets are approved in all major foreign markets (Canada, Mexico, EU, Russia, Japan, China, South Korea, Singapore, Philippines, Australia, New Zealand and Colombia). The plant tissue, or pulp, from glyphosate resistant sugarbeets is highly desirable for use as cattle feed sold in the U.S. and is readily accepted in Europe and Japan.

3. *Assuming that refined sugar produced from bioengineered sugarbeets contains genetic material is not consistent with international precedents*

Japan, China, Australia, New Zealand, Thailand, Indonesia, Malaysia, and S. Korea have strict labeling regimes, but because sugar extracted from a bioengineered sugarbeet does not contain transgenic DNA or protein, there is no requirement to label it. Indeed, as noted above, the Japanese government conducted a study that found that plant DNA is not present in the final sugar product and therefore does not include sugar produced from bioengineered sugarbeets in Japan's mandatory GMO labeling requirements.[13] Similarly, sugar produced from bioengineered sugarbeets is not included in Australia/New Zealand's mandatory GMO labeling laws because of the absence of DNA and protein in the sugar.[14] China excludes from its labeling requirements "various" highly refined products, including sugar produced from bioengineered sugarbeets.[15]

In Thailand, the Ministry of Public Health lists 22 food products which are subject to labeling requirements when the contents exceed the five percent tolerance threshold. Sugar is not included on the list.[16] Indonesia's food registration procedures require labeling for food containing genetically modified potatoes, soybeans, corn, and their derivative products. However, product derivatives which have undergone further refining processes to the point where the GE material cannot be identified (to include but not limited to oils, fats, sucrose, and starch) do not require any non-GMO statements.[17] In Malaysia refined foods, defined as those

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[13] In Japan, processed foods that contain detectable amounts of transgenic DNA or proteins must be labeled to indicate that genetically modified ingredients are used. Japan does not require sugar from GE sugarbeets s to be labeled because it does not contain transgenic DNA or proteins. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf;

[14] Under Australia New Zealand Food Standards Code - Standard 1.5.2 - Food Produced Using Gene Technology, genetically modified food or ingredients must be labeled if novel DNA and/or protein are present in the final food and also where the food has altered characteristics. In its assessment of transgenic sugarbeets, the government found that there was "no novel protein . . . present in the refined sugars, derived from sugarbeet line H7-1" and that "[i]t is unlikely that novel DNA would be present either." Thus, sugar produced from transgenic sugarbeets is not subject to the mandatory labeling requirements. *See* Food Standards Australia New Zealand Final Assessment Report Application A525 Food Derived from Herbicide-Tolerant Sugarbeet H7-1 (25 May 2005) pages 5-6 *available at* http://www.fao.org/fileadmin/user_upload/gmfp/docs/A525%20GM%20Sugar%20beet%20FAR.pdf.

[15] *See* USDA FAS, "China-Agricultural Biotechnology Annual, China Moves Towards Commercialization of Its Own Biotechnology Crops, https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Beijing_China%20-%20Peoples%20Republic%20of_12-16-2016.pdf .

[16] *See* USDA GAIN Report No. TH6136, Thailand Biosafety Act, available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Bangkok_Thailand_11-16-2016.pdf.

[17] *See* USDA GAIN Report No. 1526, Indonesian National Biosafety Commission for Genetically Engineered Products, available at

where processing has removed all novel DNA and protein, are not included in the labeling requirements (refined oil, sugar, corn syrup, honey and dextrin).[18] Finally, South Korea recently expanded their labeling law but does not include refined products such as cooking oil, sugar, soy sauce, etc.[19] No supporting documentation is required for the listed products.

4. Requiring disclosure for beet sugar when it does not contain genetic material is false and misleading, not supported by the evidence before the Agency, and will only lead to consumer confusion

Requiring all beet sugar to be disclosed as a "bioengineered" food would be false and misleading because as shown above, it does not meet the definition of a bioengineered food under the Act regardless of what threshold AMS may establish. ***Importantly, mandating that beet sugar is subject to the Disclosure Standard would represent to consumers that the beet sugar is somehow different, less safe, and less desirable than conventional beet sugar or organic or conventional cane sugar when it is molecularly identical.*** *See e.g., Center for Food Safety v. Vilsack*, 636 F.3d 1166, 1170 (9th Cir. 2012) ("The sugar produced from Roundup Ready sugarbeets is identical to sugar processed from conventional sugarbeets, and has been approved for food safety in the United States and the European Union."). As discussed above, analysis of transgenic and conventional sugarbeets and sugarcane from around the world found no plant DNA in any samples, confirming that the sugar clarification process effectively removes *all* plant DNA (by a factor of 10^{14}).

AMS should not pursue this approach because as shown above, it runs counter to the scientific evidence and contravenes Congress's intent that "USDA's implementing regulations treat the safety of a bioengineered food the same as its non-bioengineered counterpart."[20] *See Motor Vehicle Mfrs. Ass'n of U.S. v. State Farm Mut. Auto. Ins. Co.*, 463 U.S. 29, 43 (1983) (an agency's decision is arbitrary or capricious if it runs counter to the evidence before the agency, relies on factors which Congress did not intend, and/or is not otherwise the product of reasoned decision making.).

In addition, labeling beet sugar as a bioengineered food, when it does not meet the statutory definition of a bioengineered food, *misbrands* beet sugar within the meaning of the Food, Drug and Cosmetic Act. However, Congress prohibited the Disclosure Standard from affecting any

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https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_7-14-2015.pdf.

[18] *See* USDA GAIN Report No. MY6005, Malaysia Biosafety Law, the National Biosafety Board, available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Kuala%20Lumpur_Malaysia_9-2-2016.pdf.

[19] *See* USDA GAIN Report No. KS1716, Korea's New Biotech Labeling Requirements, available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Update%20to%20Korea's%20New%20Biotech%20Labeling%20Requirements_Seoul_Korea%20-%20Republic%20of_6-23-2017.pdf.

[20] Legislative History at 4.

other federal definition, program, rule, or regulation.[21] AMS must remember the Disclosure Standard is a marketing standard, which requires disclosure when the bioengineered genetic content of a food exceeds an established threshold, and is specifically not a health, safety, or nutrition standard, which the general public is unlikely to understand. Therefore, AMS must be extremely cautious to avoid any mandated disclosures that imply differences between foods when none exist.

5. ***Requiring beet sugar to be labeled as a bioengineered food also contravenes the Act's limitation that a bioengineered food is one for which the modification could not otherwise be achieved through conventional breeding and is found in nature***

AMS should not arbitrarily mandate that beet sugar is subject to the Disclosure Standard because it can be shown that the event used to confer glyphosate resistance in the sugarbeet (H7-1 conferring glyphosate tolerance; Roundup Ready™), *can also be obtained through conventional breeding methods* and is *found throughout nature*. Glyphosate tolerant sugarbeets were developed using bioengineering not based on the fact that it was the only breeding method able to create glyphosate tolerance, but rather based on the speed and accuracy with which the trait could be introduced.

All plants and microbes naturally contain a gene encoding for 5-enolpyruvylshikimate 3-phosphate synthase (a.k.a. EPSP synthase), a shikimate pathway enzyme producing aromatic amino acids in the plant essential to life. The native, or endogenous, form of this gene creates an enzyme that glyphosate binds to which inhibits functionality, killing the plant.[22] Commercially available glyphosate tolerant crops were created using bioengineering to introduce a slightly modified version of the native gene derived from *Agrobacterium tumefaciens* strain CP4.[23] The only difference between the native and transgenic version of the gene is a slight mutation which changes the 100th amino acid in the protein sequence from a Glycine to an Alanine that no longer allows glyphosate to bind to or inhibit the enzymatic pathway, conferring plant survival.[24]

Since only a mutation to the existing gene is necessary, the conventional breeding method known as mutagenesis could also have been used to create this trait in the sugarbeet. In fact, physical and chemical mutagenesis has been used to create the trait in corn.[25] It should be noted, initial attempts at mutagenesis were difficult, lengthy and often unstable. However, by 2006, Konzak

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[21] Under the Food, Drug and Cosmetic Act a food is misbranded if "its labeling is false or misleading in any particular." 21 U.S.C. § 343(a). *See also* Legislative History at 6 ("Congress does not intend the legislation to impact the authorities or obligations under the Federal Food, Drug, and Cosmetic Act, . . .").

[22] *See* Funke , T., *et al*., Molecular basis for the herbicide resistance of Roundup Ready crops, 103 *PNAS* 35 (August 29, 2006), 13010-13015.

[23] *See* Padgette, S.R., *et al*., Development, identification, and characterization of a glyphosate-tolerant soybean line, 35 *Crop Sci*. 5 (1995), 1451-1461.

[24] *See* Funke , T., *et al*., *supra* n. 22.

[25] *See* ZHAO, J., *et al.* Selection of Glyphosate-resistant Maize Mutants by Mutagenesis, 4 Journal of Henan Agricultural Sciences (2011).

and Rice filed a patent (US20070136837 A1) outlining mutagenesis protocols that produce glyphosate tolerant plants without the use of bioengineering. They demonstrated the effectiveness of the approach by creating glyphosate tolerant wheat through mutagenesis technology. Glyphosate tolerance can also be created by selection for increased expression of the EPSP synthase using conventional breeding techniques as demonstrated for carrot, alfalfa, tobacco and soybean.[26] Both of these modes of action would be equally as efficacious in sugarbeet and therefore the trait used in bioengineered sugarbeets could have been created through conventional breeding methods, although mutagenesis would take longer than bioengineering.

In addition, AMS must consider that glyphosate tolerance is found in nature. It was originally thought that glyphosate resistance would be unlikely to naturally evolve in plant populations. However reports began emerging in the late 1990s that resistance was the result of a gene mutation within EPSP synthase, a similar mode of action as glyphosate tolerant sugarbeet made through bioengineering. As more tolerance in native populations was observed, there was further confirmation that EPSP synthase was naturally mutated, reducing binding efficiency of glyphosate as well as translocation of the herbicide, both conferring resistance.[27] These and the overexpression of EPSP synthase have all now been described in nature, as well as created through conventional breeding. In fact, some plants were confirmed to be naturally resistant to glyphosate even without selective pressure.[28] Today, this natural evolution of glyphosate tolerance, especially in the presence of selective pressure from the herbicide is widely recognized throughout the published literature. Fortunately for the farmers relying on this technology at the commercial scale, effective management strategies to control resistance development in their weed species exist. Sugarbeet farmers were aware of the weed resistance issues before glyphosate tolerant sugarbeets were deregulated in 2005 and proactively took steps to use different herbicides in the fields before and after their sugarbeet crop to avoid herbicide resistance.

6. ***Requiring beet sugar to be labeled as a bioengineered food when it does not contain genetic material imposes unnecessary regulatory burdens resulting in less competition and higher consumer prices and harms the American farmer.***

The legislative history of the Act makes clear that "the Secretary, when determining the amounts of a bioengineered substance that may be present in food, or the threshold requirement, shall *minimize the impacts on all aspects of the domestic and international value chain*."[29] Moreover, the Act "is not intended to increase the costs of food manufacturing or changes in distribution or

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[26] *See e.g.,* Yu-Yau, Jo., *et al.,* Glyphosate selected amplification of the 5-enolpyruvylshikimate-3-phosphate synthase gene in cultured carrot cells, 232 *Molecular and General Genetics MGG 3* (April 1992) 377-382.

[27] *See* Powles, S. and Preston, C., Evolved Glyphosate Resistance in Plants: Biochemical and Genetic Basis of Resistance, 20 *Weed Tech.* 2 (2006) 282-289.

[28] *See* Chiou-IngYuan, *et al.*, Triple mechanisms of glyphosate-resistance in a naturally occurring glyphosate-resistant plant *Dicliptera chine,* 163 *Plant Sci.* 3 (2002) 543-54.

[29] Legislative History at 3.

handling." Congress's intent that the Disclosure Standard not disrupt domestic and international supply chains is reinforced by E.O. 13777, which established a federal policy to alleviate unnecessary regulatory burdens. The Department of Agriculture recently requested public comment on how its Task Force, required by E.O. 13777, can reduce the regulatory burdens of existing regulations, particularly regulations that are unnecessary, impose costs that exceed benefits, or eliminate jobs. 82 Fed. Reg. 32649 (July 17, 2017). The same principles apply to new regulations.

Requiring beet sugar to be labeled as bioengineered foods when it does not contain genetic material exacerbates the impacts on the domestic and international value chain:

a. It discriminates against beet sugar by implying to consumers that it is different or less desirable than conventional beet sugar and organic and conventional cane sugar when it is molecularly identical to these other refined sugars. This leads to price differentiation, with premiums imposed for the "more desirable" products and aggressive marketing to gain market share. Already the Non-GMO Project label on some cane sugar brands and cane sugar-containing products is being used to suggest to consumers that cane sugar and products containing it are more desirable than beet sugar.

b. Any time identical products are differentiated in the market it causes food manufacturers and retailers to restrict their supply chain thereby reducing competition and driving up costs which are eventually passed onto consumers through higher prices. This was clearly evidenced in 2015-2016 as food manufacturers began to constrict their supply chains in order to comply with the Vermont law.

c. Large nationwide retailers will source sugar from multiple suppliers of beet and cane that are then packaged into the retailer's house-branded packages. If disclosure requirements are different for beet and cane, then house brands would need different labels and present implied differences to consumers where none exist, resulting in higher consumer prices.

d. Through a process known in the industry as "swapping", beet and cane sugar is often sold by a particular sugar refiner but delivered to customers from competitors who are geographically closer to the competitor's customers market. This efficient system that reduces transportation costs and congestion on rails and roads, and lowers costs to consumers, would be lost.

Finally, disruption in the supply chain and disparagement of the technology harms the American sugarbeet farmer because demand for genetically engineered sugarbeets will decline, even though they improve crop yields and are more environmentally sustainable than conventional crops.[30] Indeed, when the Vermont law was enacted many farmers faced uncertainty regarding

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[30] *See also* "Crop biotechnology has contributed to significantly reducing the release of greenhouse gas emissions from agricultural practices. This results from less fuel use and additional soil carbon storage from reduced tillage with GM crops. In 2012, this was equivalent to removing 27 billion kg of carbon dioxide from the atmosphere or equal to removing 11.9 million cars from the road for one year." GM crops: global socio-economic and environmental impacts 1996-2012. PG Economics Ltd, UK. http://www.pgeconomics.co.uk/page/36/-gm-crop-use-continues-to-benefit-the-environment-and-farmers.

the future viability of their bioengineered crops. AMS should be mindful that in enacting the Disclosure Standard Congress made "every effort . . . to ensure that farmers access to seed technology and not limit the options available to agricultural production" and directed USDA "to take every effort to minimize the impacts on growers."[31] Impacting the American farmer is also directly contrary to E.O. 13790, which established an interagency Task Force to "identify legislative, regulatory, and policy changes to promote in rural America agriculture, economic development, job growth, infrastructure improvements, technological innovation, energy security, and quality of life."[32] This includes advancing "the adoption of innovations and technology for agricultural production and long-term, sustainable rural development." Biotechnology is at the forefront of agricultural innovation enabling farmers to produce more food on fewer acres using less energy and fewer pesticide applications. Any mandate that refined foods that do not contain genetic material be subject to the Disclosure Standard undermines the advancement of technology for agricultural production in direct contravention of E.O. 13790.

As the world leader in bioengineered crop production, the United States should send a strong message to all nations that bioengineered seeds have significant economic and environmental benefits; the U.S. should not create a Disclosure Standard that discriminates against the technology. Requiring disclosure of refined food products not containing genetic material would only perpetuate the misinformation activists have used for decades to distort the truth about biotechnology, instilling fear in the general public when the global scientific community has repeatedly attested to its safety.[33] Indeed, in making clear that the Disclosure Standard is a marketing standard, not a health, safety, or nutritional standard, Congress expressly recognized that "the comprehensive federal regulatory review process has determined that foods produced using bioengineering are safe and not materially different in any way from those made using other methods."[34]

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[31] Legislative History at 7.

[32] See Executive Order 13790, "Promoting Agriculture and Rural Prosperity in America" https://www.federalregister.gov/documents/2017/04/28/2017-08818/promoting-agriculture-and-rural-prosperity-in-america;

[33] *See e.g.*, National Academy of Sciences, The Royal Society of Medicine, WHO, OECD, the American Medical Association, Food and Agriculture Organization of the United States, American Diabetes Association, and the Society of Toxicology.

[34] Legislative History at 4.

7. Mandating disclosure would threaten other foreign beet and cane producers that may adopt bioengineered technology in the future to improve environmental impact and sustainability.[35]

The U.S. is the third largest sugar importer in the world. The U.S. provides access to 41 countries to supply approximately 30% of our sugar market. Any effort to differentiate between beet and cane sugar would cause foreign beet and cane producers to avoid technology that would be better for the environment and increase their efficiency and productivity. This undermines global sustainability objectives.

The United States already imports sugar derived from BE sugarbeets (Alberta) and actual BE sugarbeets from Ontario, Canada for processing in Michigan. Brazil's government recently approved the world's first commercial bioengineered sugarcane modified to express Bt (Bacillus thuringiensis), which confers resistance to an insect referred to as the cane borer. Brazil is by far the largest sugarcane producer and exporter in the world and is the third largest supplier of raw sugar to the U.S. Current expectations are that sugar derived from the new variety will reach commercial export markets in 2020. As the world leader in sugarcane production, other cane producing countries look to Brazil for technical advances. For example, Australia and Indonesia are currently developing BE sugarcane varieties with drought resistance, herbicide tolerance, plant development, increased sugar content, and yield.[36] These advances will provide many environmental benefits and increase long term sustainability. Misguided labeling schemes for refined ingredients, such as sugar, should not inhibit such advances.

If sugar derived from a BE plant were required to be labeled it would also be problematic for our trade with Canada. Brazil is the largest raw sugar supplier to Canada. (7-year Olympic average is 78% of all raw imports). Canadian companies manufacture sugar-containing products for export to the United States. If the sugar derived from bioengineered crops were required to be disclosed then raw sugar imported from Brazil would have to be segregated from other raw sugars derived from non-bioengineered cane in the Canadian refineries. Also, Canada annually exports around 550,000 short tons of sugar in sugar-containing products to the United States duty free. If sugar derived from bioengineered crops would be required to be labeled, this would place unnecessary burdens on our trading partners and discourage the adoption of bioengineered crops that are more productive and environmentally sustainable.

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[35] *See* U.S. Beet Sugar Industry Submission to the National Academy of Sciences, September 9, 2015, http://www.sugarindustrybiotechcouncil.org/wp-content/uploads/2015/11/U-S-Beet-Sugar-Industry-Submission-to-NAS3.pdf. *See also* "Crop biotechnology has contributed to significantly reducing the release of greenhouse gas emissions from agricultural practices. This results from less fuel use and additional soil carbon storage from reduced tillage with GM crops. In 2012, this was equivalent to removing 27 billion kg of carbon dioxide from the atmosphere or equal to removing 11.9 million cars from the road for one year." GM crops: global socio-economic and environmental impacts 1996-2012. PG Economics Ltd, UK, http://www.pgeconomics.co.uk/page/36/-gm-crop-use-continues-to-benefit-the-environment-and-farmers.

[36] https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Canberra_Australia_8-7-2015.pdf USDA Gain Report on Australia; https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_7-14-2015.pdf USDA Gain Report on Indonesia

8. *There is no legal or scientific basis for AMS to treat beet sugar differently than fermentation products that are derived from bioengineering*

According to the Legislative History, "Congress intends the Secretary to provide exemptions and other determinations under which a food product is not considered bioengineered. Congress recognizes that states that had passed labeling mandates provided exemptions for a range of food products including … those that may include enzymes, additives, and processing aides."[37] As members of the Coalition for Safe and Affordable Food, we strongly endorse the Coalition's response to Question 11 identifying categories of foods that AMS should exempt from the Disclosure Standard. There is wide consensus that fermentation products, e.g., enzymes, processing aids, should not be subject to the Disclosure Standard solely because they are produced using a bioengineered microorganism. Even the EU, with its strict labeling regime exempts "processing aides (like food enzymes produced from GE microorganisms)."[38] This position is legally justified because a food product produced with enzymes or processing aids would not meet the definition of a bioengineered food under the Act (one that contains genetic material above the established threshold) and is scientifically substantiated using validated scientific methods.

The same legal and scientific justification applies to beet sugar. As shown above, the science substantiates that beet sugar does not contain genetic material of any kind. There is no rational basis under the Act to exempt one category of foods produced using a bioengineered organism but require disclosure for beet sugar that does not contain any genetic material.[39] Fairness dictates that all foods should be subject to the same criteria. In both cases the definition of bioengineering in the Act makes it clear that the law does not apply to products that do not contain genetic material above any threshold established by the Secretary.

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[37] Legislative History at 3.

[38] USDA Gain Report on the EU at 29, https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf

[39] To justify the disparate treatment of fermentation products and refined products some may argue that fermentation products such as microbes and processing aids are not themselves food but refined products such as sugar and oils are food. That distinction is legally and scientifically unsupportable.

QUESTION 8

What is the amount of a bioengineered substance present in a food that should make it be considered bioengineered? (Sec. 293(b)(2)(B)).

Context: *The Law authorizes the Secretary to determine the amount of a bioengineered substance present in food for the food to be disclosed as a bioengineered food. The amounts of a bioengineered substance that may be present in food for the food to be a bioengineered food might be determined in a variety of ways: if a bioengineered substance is near the top of the list of ingredients, by determining the percentage of bioengineered ingredients in a food product, or by listing any ingredient that was produced through bioengineering, among others. AMS is considering how to determine the amount of bioengineered food or ingredient needed for a product to require a bioengineered disclosure, as well as the advantages and disadvantages of various methods.*

In determining the amount of a bioengineered substance (referred to in the Act as "genetic material"), AMS identifies as one option "listing any ingredient that was produced through bioengineering." This option would be wholly inconsistent with the Act because the Congress did **NOT** intend and the Act does **NOT** apply to food or ingredients produced *through* bioengineering. Rather, the Act only applies to a "bioengineered food" which "contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature. Congress clearly recognized that there would be foods that are produced through bioengineering that would not be subject to the disclosure standard. Basing the trigger for disclosure on whether an ingredient was produced *through* bioengineering impermissibly rewrites the statutory definition of a bioengineered food and contravenes Congress's intent.

Other methods AMS may use to set the disclosure threshold are critically important and have direct implications as to how the technology is viewed by consumers and global trading partners. Thus, given its impact on the current and future use of the technology, we are compelled to offer our views. ***We strongly urge AMS to set a 5% threshold because it supports biotechnology, appropriately balances disclosure, market dynamics, and international trade, and is consistent with other U.S. regulatory programs, including the USDA Organic Program which allows up to 5% of non-organically produced agricultural ingredients.*** Like the USDA, we have conducted extensive research on bioengineering disclosure methods worldwide and provide the following observations.[40]

It should be clearly understood that there is no international standard for bioengineered thresholds. Nor is there any scientific basis for the threshold percentages because biotechnology does not raise health, safety or nutrition concerns.[41] Accordingly, thresholds are simply a tool to

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[40] *See* Attachment 2 ("Bioengineered Disclosure Thresholds").

[41] *See e.g.,* USDA Foreign Agricultural Service, European Union 28, Agricultural Biotechnology Annual, December 6, 2016 at 20, 37 (noting that "the EC continues to pursue inconsistent and unpredictable approaches regulating the

create a differentiation in the market place to provide a marketing advantage to non-bioengineered products. Thresholds are arbitrarily established mainly to drive consumers away from the technology and create non-tariff trade barriers to imported biotech commodities to protect domestic producers who do not have access to the technology.[42] As a world leader, and a leader in biotechnology, the U.S. must set its threshold standard on multiple justifications and not acquiesce to standards set by other countries that attempt to oppose or stigmatize the technology. It is also important to keep in mind that "Congress intend[ed] for the standard to be technology neutral."[43] Other countries are closely watching what the U.S. will do in these regulations and it will likely influence their internal discussions regarding acceptance and disclosure.

International thresholds for disclosure of bioengineered foods can be categorized into three groups:

> **Approach 1** is to treat bioengineered ingredients as no different than other ingredients and not have any mandatory labeling requirements. There are 116 countries (including neighboring trading partners, Canada and Mexico), representing 59% of the countries in the world and 24% of the world population, following this approach. This approach indicates support, trust, acceptance and fostering of bioengineering and bioengineered crop ingredients. This results in lower ingredient costs, greater savings to consumers, provides multiple environmental benefits, does not impact the domestic and international value chain, and is technology neutral. This should be the global standard. However, after two decades of activists maligning the technology and costly state-by-state labeling referendums, Congress responded by enacting the Disclosure Standard. Therefore, this approach is no longer available to the U.S.

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technology. Due to the strong emotional and ideological stance taken by EU consumers and nongovernmental organizations (NGOs) on biotechnology, born in many ways out of the misleading information provided by anti-biotechnology groups, legislation adopted by the EC as well as the process surrounding the approval for cultivation and use of GE crop varieties has suffered," and further noting that "different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage . . . and communication campaigns to heighten public fears."), available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf;

[42] The European Union's moratorium on approving new genetically modified food illustrates the point. In 2003, the U.S., Canada, and Argentina challenged the moratorium as unfair protectionist measures prohibited by the General Agreement on Tariffs and Trade (GATT). The Panel concluded that "the European Communities applied a general de facto moratorium on approvals of biotech products between June 1999 and 29 August 2003." *See* European Communities – Measures Affecting the Approval and Marketing of Biotech Products. WTO Document WT/DS291R (29 September 2006).

[43] Legislative History at 4.

Approach 2 is to treat bioengineered ingredients as a non-disparaged low-level presence ingredient. Some countries that follow this approach have a 5% threshold, including Japan, South Africa, Indonesia, Vietnam, and Thailand (collectively representing 8% of the world population). Canada has a voluntary 5% threshold.

Approach 3 is to treat bioengineered ingredients as contaminants. Countries (EU, China [44], Russia, etc.) following Approach 3 have thresholds that range from 4% to 0.0% and outright bans. For example, Nigeria has a 4% threshold;[45] Malaysia and Taiwan (not recognized as a country) have a 3% threshold; Brazil, Australia, New Zealand, Saudi Arabia have a 1% threshold; 41 countries have a 0.9% threshold;[46] 21 countries representing 43% of the world population have a 0.0% threshold;[47] and Kenya, Morocco, Benin, Sri Lanka, and Serbia have outright bans.[48] It is important to note that there is clear evidence that a low threshold in one country has a direct and dramatic negative impact on the acceptance of biotechnology by other countries. The EU's 0.9% threshold that has existed for some time has severely restricted the use of biotechnology within the EU and also with its trading partners who supply the EU with raw agricultural products and finished food products.

We urge AMS to adopt a 5% threshold (Approach 2) and demonstrate its leadership on biotechnology

Of the thresholds that have been established world-wide, a 5% threshold is the most supportive of bioengineering. It is the lowest cost, lowest liability approach that results in consumer savings. It also has the least impact on the domestic and international value chain and is less of a burden

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[44] "In September 2014, the government released remarks by President Xi Jinping affirming official support for biotechnology research, but calling for a cautious approach to commercialization. He also said that foreign companies should not be allowed to "dominate the agricultural biotechnology product market." Page 2, USDA Foreign Agricultural Service, China, Agricultural Biotechnology Annual, December 16, 2016, https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Beijing_China%20-%20Peoples%20Republic%20of_12-16-2016.pdf . The pending acquisition of Syngenta by ChemChina may facilitate a greater acceptance of biotechnology.

[45] Nigeria enacted the Biosafety Act in 2015 that requires mandatory labeling of all products of agricultural biotechnology. Work in progress regulations have a 4% threshold.

[46] These include the 28 EU Member States, Russia, Ecuador, Iceland, Norway, Switzerland, Turkey, Ukraine, Botswana, Bosnia and Herzegovina, Belarus, Kazakhstan, Armenia, Kyrgyzstan).

[47] These include China, Peru, Columbia, Bahrain, Kuwait, Oman, Qatar, United Arab Emirates, South Korea, Ethiopia, Cameroon, India, Mozambique, El Salvador, Bolivia, Tunisia, Mauritius, Burkina Faso, Senegal, Mali, and Bangladesh.

[48] "Morocco's heavy reliance on the EU market as the principal destination for its agricultural exports has instilled a reluctance among policy makers and producers to accept biotechnology products." Morocco, Agricultural Biotechnology Annual, 2016, USDA Foreign Agricultural Service, Global Agricultural Information Network (GAIN Report Number MO1610), https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Rabat_Morocco_11-7-2016.pdf.

on our developing foreign suppliers. It is the most compatible with our North American trading partners, Mexico and Canada. Finally, it is the closest to technology neutral of the mandatory categories.

Importantly, a 5% threshold is consistent with other U.S. regulatory programs. The USDA Organic Program allows up to 5% of non-organically produced agricultural ingredients which are not commercially available in organic form.[49] "The use of genetic engineering, or genetically modified organisms (GMOs), is prohibited in organic products."[50] However, "[t]here aren't specific tolerance levels in the USDA organic regulations for GMOs. As such, National Organic Program policy states that trace amounts of GMOs don't automatically mean the farm is in violation of the USDA organic regulations. In these cases, the certifying agent will investigate how the inadvertent presence occurred and recommend how it can be better prevented in the future."[51] If an organic consumer product can retain the organic label with up to 5% non-organic content, the Disclosure Standard should be set at 5% as well. Indeed, federal courts have held that consumers hold products labeled organic to a higher standard than even products labeled natural. *See e.g., Pelayo v. Nestle USA Inc.*, 989 F. Supp. 2d 973, 979 (C.D. Cal. 2015). Having the same 5% threshold reduces consumer confusion and avoids any implication that biotechnology is less safe or less desirable and therefore must be treated more stringently than organic products. In addition, the grain trade has coalesced around a 5% low-level presence threshold, although there isn't an international standard.

Establishing a threshold below 5% (Approach 3), as many groups will urge, denigrates biotechnology

We implore AMS to keep Congress's intent in mind that "[n]othing in the [disclosure] requirement can be used to denigrate biotechnology."[52] Approach 3, is not supportive of bioengineering or bioengineered foods, crops or biotechnology. For over 20 years the U.S. has battled foreign countries that inhibit or reject U.S. exports because of their overly restrictive biotechnology standards, based principally on fear (the precautionary principle), not science.[53]

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[49] USDA Labeling Organic Products, https://www.ams.usda.gov/sites/default/files/media/Labeling%20Organic%20Products.pdf.

[50] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[51] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[52] Legislative History at 2.

[53] See also "In the EU, different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage (destruction of research trials and cultivated fields), and communication campaigns to heighten public fears." Page 37, USDA Foreign Agricultural Service, European Union 28, Agricultural

This has resulted in higher food costs to foreign consumers and less sustainable food production. In many instances, these restrictive thresholds are used as a non-tariff trade barrier to imports to protect their domestic producers from U.S. competition.

Adopting a threshold of less than 5% would complicate our trade with our major and neighboring trading partners, Canada and Mexico, neither of which require any disclosure. As the legislative history directs, "…the Secretary, when determining the amounts of a bioengineered substance that may be present in food, or the threshold requirement, **shall minimize the impacts on all aspects of the domestic and international value chain**."[54] Any threshold of less than 5% maximizes impacts to all aspects of the domestic and international value chain.

Moreover, the Non-GMO Project, whose stated mission is to "to change the way our food is grown and made," has a 0.9% per ingredient threshold above which a product cannot bear its Non-GMO Project verified label.[55] That is not Congress's intent. Congress made clear that the Disclosure Standard cannot "denigrate biotechnology," which is precisely the Non-GMO Project's undeniable objective in order to drive bioengineered foods out of the market. To adopt the same threshold used by the Non-GMO Project is unsupportable and unacceptable to the American farmers that embrace biotechnology.

In sum, USDA will determine whether the United States will continue to treat the presence of bioengineered substance in food as a "non-disparaged low-level presence ingredient" or a "contaminant." It is our belief that the only threshold that will allow the United States to remain a world leader in the production of bioengineered crops and minimizes impacts on the value chain, minimizes the regulatory burden on farmers, is a 5% threshold. When a food product contains over 5% of ingredients that are bioengineered, this should be disclosed to consumers to inform their purchasing decisions. Any lower threshold would treat bioengineered ingredients as a contaminant and not be technology neutral and would "denigrate biotechnology" in contradiction of Congress.[56]

Biotechnology Annual, December 6, 2016. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf.

[54] *Id.*

[55] Non-GMO Project, https://www.nongmoproject.org/about/mission/.

[56] Legislative History at 2.

QUESTION 9

Should AMS consider more than one disclosure category? (Sec. 293(b)(2)(D))

Context: *AMS is considering if it should develop various categories for disclosure and if it should differentiate between those products that a) are bioengineered, b) contain ingredients that are bioengineered, or c) contain ingredients derived from bioengineered crops or animals. Additionally, AMS is considering the creation of a set of disclosures for a category of bioengineered foods for those products that, due to changes in sourcing, include bioengineered ingredients for part of the year, and non-bioengineered ingredients for other parts of the year. AMS is considering the advantages and disadvantages, based on cost, clarity, and other factors, of using a single disclosure category or multiple disclosure categories.*

The law creates two categories for disclosures: bioengineered foods and foods that may be bioengineered. We urge AMS to adhere to the statutorily prescribed categories.

Under no circumstances should AMS create a category of disclosure for foods that "contain ingredients derived from bioengineered crops or animals," As set forth in our comments on Question 4, such a disclosure category would be contrary to the plain language and intent of the Act and would exceed AMS's authority. The determining factor for whether the Act applies to a food is not the breeding method by which a food was derived but the "presence of genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature" above an amount determined by the Secretary.

It is unrealistic for a company to change labels every time it changes ingredients between bioengineered and conventional commodities. For a category of bioengineered foods for those products that, due to changes in sourcing, include bioengineered ingredients for part of the year, and non-bioengineered ingredients for other parts of the year, a single label noting "may contain bioengineered ingredients" can account for different sourcing throughout the year of bioengineered and non-bioengineered crops.

QUESTION 10

What other factors or conditions should AMS consider under which a food is considered a bioengineered food? (Sec. 293(b)(2)(C))

Context: *AMS must develop a process to help stakeholders determine whether a food is subject to bioengineered disclosure. AMS anticipates the process would include considering factors such as these: whether a food contains a substance that has been modified using recombinant in vitro DNA techniques (Sec. 291(1)(A)), and for which the modification could not be obtained through conventional breeding or found in nature (Sec. 291(1)(B); Question 2 and 3), , and whether a food requires disclosure based on the predominance of ingredients (Sec. 292(c), Question 6), among others. The outcomes of these determination requests might be publicly posted on a Web site. The process to implement Sec. 293(b)(2)(C) is not intended to be an investigation or enforcement process (see Questions 26-29); instead, the implementation would likely be framed for manufacturers or developers of bioengineered food or ingredients who have a question on whether their food is subject to disclosure. AMS is considering the factors to be considered, the way to inform the public about the outcome of the requests, and ideas regarding the process to be used to make the determination.*

We agree that it would be helpful for AMS to establish a process for manufacturers or developers of bioengineered food or ingredients to seek clarification on factors that should be considered in determining whether a food meets the definition of a bioengineered food. However, as stated throughout this comment, any determinations made in response must adhere to the statutory definition of a bioengineered food, one that "contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature" above an amount determined by the Secretary. Section 293(b)(2)(C) is not a broad grant of authority that allows AMS to rewrite the definition of a bioengineered food. Thus, whatever factors AMS considers, they cannot modify the definition of a bioengineered food as one that contains bioengineered genetic material.

We also recommend that AMS, as part of the § 293(b)(2)(C) process, allow manufacturers to seek a confirmation that a food is not bioengineered within the meaning of the Act. Such a mechanism would be consistent with APHIS's current "Am I regulated" letter of inquiry process that allows biotechnology developers to inquire as to whether a genetically engineered organism is regulated under the Plant Protection Act. AMS could, as APHIS does, make these determinations public which would further help clarify those foods that are not subject to the Disclosure Standard. It would also be consistent with Congress's intent that "the Secretary . . . provide exemptions *and other determinations* under which a food is not considered bioengineered,"[57] as well as with other countries (Japan, China, Australia, New Zealand,

[57] Legislative History at 3.

Thailand, Malaysia, S. Korea, and Indonesia), that specifically recognize that certain foods, such as sugar are not bioengineered.[58]

[58] Japan exempts sugar from GE sugarbeets from their GE labeling requirements. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf; in China, sugar derived from GE sugarbeets and various other refined foods, e.g., cottonseed oil, are not subject to mandatory labeling, "China-Agricultural Biotechnology Annual, China Moves Towards Commercialization of Its Own Biotechnology Crops, https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Beijing_China%20-%20Peoples%20Republic%20of_12-16-2016.pdf; Australia exempts sugar derived from GE sugarbeets and other foods that do not contain any novel DNA or protein from its labeling laws, Food Standards, Australia New Zealand, http://www.foodstandards.gov.au/consumer/gmfood/labelling/Pages/default.aspx.

QUESTION 12

If a manufacturer chooses to use text to disclose a bioengineered food, what text should AMS require for a text disclosure? (Sec. 293(b)(2)(D))

Context: *Currently, some food manufacturers use language compliant with the Consumer Protection Rule 121 from the State of Vermont to identify their food products as bioengineered ("Produced with Genetic Engineering," "Partially Produced with Genetic Engineering," or "May be Produced with Genetic Engineering"). AMS is considering whether to allow manufacturers to continue using these disclosures under the new national bioengineered disclosure standard and if their language is appropriate. Further, AMS is considering what phrases could be used as a text disclosure for bioengineered food that consumers would find informative, truthful, and not misleading. AMS is also considering whether there should be one standard text disclosure language, or whether manufacturers should be allowed flexibility to choose from more than one acceptable phrase and where the bioengineered food disclosure should be placed on food packages.*

We offer the following recommendations:

1) AMS should not allow manufacturers to continue using the disclosures established under the Vermont law most importantly because the Vermont law disclosures conflict with the plain language and intent of the Act. The Vermont disclosures have highly restrictive thresholds and include food ingredients that are derived from but do not contain genetic material. While such disclosures may have been consistent with Vermont's unfounded health, safety, and nutritional concerns, Congress expressly rejected Vermont's approach and instead defined bioengineering with respect to a food as one that contains genetic material. Thus, adhering to Vermont's prescribed disclosure language ("Produced with Genetic Engineering," "Partially Produced with Genetic Engineering," or "May be Produced with Genetic Engineering") cannot be reconciled with the Act. Further, adhering to this language would be misleading because it would imply differences in certain food products when none exist. For the many reasons stated in response to question 4, any language that includes "produced from," "derived from" or "sourced from" is unacceptable when the ingredient provided to the consumer is no different than an ingredient derived from a conventional or organically grown crop.

2) We also urge AMS not to allow the use of "May be Produced with Genetic Engineering". First, the "may be" language is ambiguous and therefore creates a perception that the food manufacturer is uncertain about a product's ingredients. Second, "produced with" implies that the food is "derived from" or "sourced from" a bioengineered crop, contrary to the intent of the Act. Third, the term "genetic engineering" is broader than and therefore inconsistent with the Act's definition of bioengineering. Similarly, "Partially Produced with Bioengineering" is incorrect because it implies that the food is "derived from" or "sourced from" a bioengineered crop.

3) The terminology that we urge AMS to use is "*contains* bioengineered ingredients" or "may *contain* bioengineered ingredients." These statements are informative, truthful, and not misleading. They also adhere to the Act's definition of bioengineering and would not require manufacturers to change labels when they change sources between bioengineered and non-bioengineered ingredients. (*See* Question 9 at 21).

4) We urge AMS to adopt one standard text disclosure language to fulfill the Act's purpose to establish uniformity in disclosure. As AMS is well aware, there are many terms used to describe whether a food is or is not bioengineered, most of which are not accurate nor well understood by the general public. We believe uniformity is best accomplished and consumer understanding advanced by limiting on package text to "contains bioengineered ingredients" or "may contain bioengineered ingredients."

5) Just as important as the text, is the font size and location on the package. For consumers who want to know what is in their food, the information is located on the Nutrition Facts Panel, the ingredient list and the allergy warnings, all under FDA's authority. Any information about bioengineered ingredients or non-bioengineered ingredients should be located as close to the ingredient list as possible, but not in a font size larger or more prominent than the allergy warnings which is alerting consumers that the food contains an allergen that can be harmful or fatal to sensitive individuals. Non-GMO labeling efforts attempt to imply to consumers that a product is safer, healthier or more nutritious than other products derived from biotechnology, which is false and misleading. Therefore, all text information or symbol regarding bioengineered food should be located in close proximity to the ingredient list and allergy warning in a font size that does not exceed that information. The legislative history also provides guidance in this area, stating: "Congress intends USDA to establish any text or the symbol that could appear on packaging to solely satisfy the disclosure requirement and not be used as a tool to denigrate biotechnology."[59] Giving the on-package disclosure more prominence than allergy warnings would potentially denigrate biotechnology.

[59] Legislative History at 3.

QUESTION 23

Is there other equivalent on-package language that AMS should consider to accompany an electronic or digital disclosure besides "Scan here for more food information"? (Sec. 293(d)(1)(A)

Under no circumstances should the text accompanying an electronic or digital disclosure reflect that a food may or may not be bioengineered. Congress purposely directed that text accompanying the electronic or digital disclosure be limited to "scan here for more food information" or equivalent language "that only reflects technological changes." § 293 (d). Congress was rightfully concerned that any text relating to bioengineering would equate to *de facto* on package labeling which Congress expressly rejected.

To address the concern that the word "scan" may not be relevant as technology changes in the future we suggest that equivalent language could be "Access more food/ingredient information here." This would alert the consumer that some further action was required to obtain more information about the food.

QUESTION 30

What should the requirements for imports into the United States of products covered by the Law/regulation be? (Sec. 294(a))

Context: *AMS considering how the disclosure requirements should be applied to imported products*

Imported products should be required to follow the same disclosure requirements as products manufactured in the United States. The U.S. should allow stickers or stamps to be used for any required disclosures before import and customs clearance to assure compliance and eliminate the risk of liability to U.S. entities throughout the distribution chain. The U.S. should apply any requirements in a nondiscriminatory way that is consistent with U.S. obligations under World Trade Organization and other international trade and investment agreements.

As outlined in the response to Question 4, the United States already imports sugar derived from BE sugarbeets (Alberta) and actual BE sugarbeets from Ontario, Canada for processing in Michigan. Brazil's government recently approved the world's first commercial bioengineered sugarcane that contains the gene Bt (Bacillus thuringiensis) that is resistant to an insect referred to as the cane borer. Brazil is by far the largest sugarcane producer and exporter in the world and is the third largest supplier of raw sugar to the U.S. Current expectations are that sugar derived from the new variety will reach commercial export markets in 2020. As the world leader in sugarcane production, other cane producing countries look to Brazil for technical advances. For example, Australia and Indonesia are currently developing BE sugarcane varieties with drought resistance, herbicide tolerance, plant development, increased sugar content, and yield.[60] These advances will provide many environmental benefits and increase long term sustainability. Misguided labeling schemes for purified ingredients, such as sugar, should not inhibit such advances.

If sugar derived from BE sugarbeets were required to be labeled it would also be problematic for our trade with Canada. Brazil is the largest raw sugar supplier to Canada. (7-year Olympic average is 78% of all raw imports). Canadian companies manufacture sugar-containing products for export to the United States. If the sugar derived from bioengineered crops were required to be disclosed then raw sugar imported from Brazil would have to be segregated from other raw sugars derived from non-bioengineered cane in the Canadian refineries. Also, Canada annually exports around 550,000 short tons of sugar in sugar-containing products to the United States duty free. If sugar derived from bioengineered crops would be required to be labeled, this would place unnecessary burdens on our trading partners and discourage the adoption of bioengineered crops that are more productive and environmentally sustainable.

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[60] https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Canberra_Australia_8-7-2015.pdf USDA Gain Report on Australia; https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_7-14-2015.pdf USDA Gain Report on Indonesia

ATTACHMENT 1

ELSEVIER

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Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets

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Abstract

The fate of cellular DNA during the standard purification steps of the sugar manufacturing process from conventional and transgenic sugar beets was determined. Indigenous nucleases of sugar beet cells were found to be active during the first extraction step (raw juice production) which was carried out at 70°C. This and the consecutive steps of the manufacturing process were validated in terms of DNA degradation by competitive PCR of added external DNA. Each step of the process proved to be very efficient in the removal of nucleic acids. Taken together, the purification steps have the potential to reduce the amount of DNA by a factor of $>10^{14}$, exceeding by far the total amount of DNA present in sugar beets. Furthermore, the gene products of the transgenes neomycin phosphotransferase and BNYVV (rhizomania virus) coat protein CP21 were shown to be removed during the purification steps, so that they could not be detected in the resulting white sugar. Thus, sugar obtained from conventional and transgenic beets is indistinguishable or substantially equivalent with respect to purity. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Beta vulgaris*; Transgenic sugar beets; Sugar purification; Competitive PCR; Rhizomania

1. Introduction

The development of transgenic varieties of various plants, and also sugar beets, had become feasible by application of selectable marker gene introduction with the Ti-plasmid derived vectors due to the pioneering work of Bevan et al. (1983) and Herrera-Estrella et al. (1983). For the generation of transgenic sugar beets (*Beta vulgaris*), an improved method using stomatal guard cells has recently been reported (Hall et al., 1996). Since then, numerous transgenic lines have been constructed and their usefulness demonstrated in outdoor plantations.

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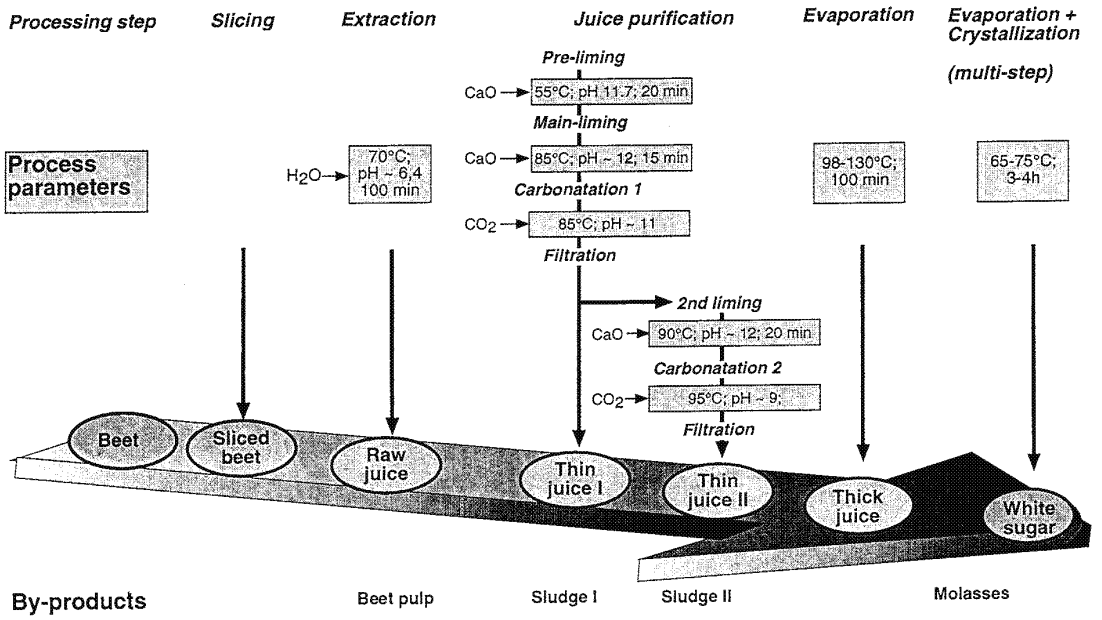

Fig. 1. Principal steps of sugar production from sugar beets.

One major goal in generating transgenic varieties is the establishment of resistance against plant viruses. The first report on this made use of the introduction and expression of virus coat protein genes in plant cells (Abel et al., 1986). The major virus related disease of sugar beets is rhizomania caused by the beet necrotic yellow vein virus (BNYVV). The genetic map of the multipartite genome of this virus has been reviewed (Richards and Tamada, 1992). The introduction of a gene cassette coding for the *cp*21 gene product (coat protein, CP21) of BNYVV under the control of the cauliflower mosaic virus promoter into cells of *B. vulgaris* resulted in plants resistant to BNYVV infection (Kallerhoff et al., 1990; Ehlers et al., 1991). The addition of this gene cassette to the genome of *B. vulgaris* was supported by coupling the CP21 construct to a neomycin resistance gene (*aphA*) allowing selection by G418 treatment of cultivars during the early stages of their cultivation.

The first successful outdoor plantations of transgenic virus resistant sugar beet cell lines raised the question about the fate of genetic material and proteins during the sugar manufacturing process.

Sugar is recovered from beet by a multistep extraction and purification procedure (Fig. 1). This includes slicing of washed beets (to 'cossettes') followed by extraction with water at elevated temperature (70°C) for about 100 min. The raw juice obtained is clarified by two consecutive steps comprising CaO addition (liming) and subsequent carbonatation. The material precipitated thereby (sludge) is removed by filtration to yield a so-called thin juice. It is concentrated by evaporation first to thick juice and then further to a crystal magma from which high purity sugar is recovered by centrifugation. The evaporation of thin juice to thick juice is carried out in a multi-effect evaporator working at a temperature range of 98–130°C.

The objective of this study was to analyse intermediate and end products of the standard sugar recovery process for DNA using the ADP-glucose pyrophosphorylase gene (AGPase, *agp*, Smith-

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White and Preiss, 1992) as a general marker for sugar beet DNA, and the genes for the BNYVV coat protein (*cp*21) and neomycin phosphotransferase (*aphA*) and their respective gene products as specific markers for transgenic beet DNA and proteins. Furthermore, the potential of each principle processing step to remove DNA was validated with added pUC18 DNA (Yanisch-Perron et al., 1985). The methods applied comprised agarose gel electrophoresis, hybridisation methods, competitive PCR and immunological as well as enzymatic methods.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Cloning experiments and plasmid preparations were carried out in *E. coli* JM109. Strains with plasmids were grown in 2 × YT liquid medium or on 2 × YT agar plates (Sambrook et al., 1989) supplemented with 100 μg ml^{-1} ampicillin at 37°C.

2.2. DNA preparation, DNA manipulation and cell transformation

Plasmid preparations from *E. coli* were performed by the method of Kieser (1984). Large scale plasmid preparation was done by using the Qiagen plasmid giga kit (Qiagen, Hilden, Germany). To isolate genomic DNA, frozen beets or frozen cossettes (3 g) were chopped up in liquid nitrogen and homogenised for 2 min in 1 vol Kirby mix (1% triisopropylnaphthalenesulfonic acid, Na salt, 6% 4-aminosalicylic acid and 6% phenol in 50 mM Tris–HCl, pH 8.3; Sambrook et al. (1989)) and 2 vol phenol/chloroform. After centrifugation, the supernatant was reextracted with 1 vol phenol/chloroform and the DNA precipitated with ethanol. Finally, the DNA was resuspended in TE buffer (Sambrook et al., 1989) and dialysed in the same buffer. Raw juice (1 ml), thin juices (1 ml), samples of sludge I and II (1 g resuspended in 1 ml TE buffer), thick juice (1 ml) and white sugar (3 g diluted in 3 ml water) were treated with 0.5 ml phenol/chloroform and centrifuged at 6000 × *g* for 15 min. The supernatant was dialysed in a buffer containing PEG 6000 (5 mM Tris–HCl, pH 8.8, 0.5 mM EDTA, 5 mM NaCl, 3.5% PEG 6000) and hereby 10-fold concentrated. Finally, the DNA was purified via the Qiaquick-spin PCR purification kit of Qiagen. All other DNA manipulations were carried out as described elsewhere (Sambrook et al., 1989).

2.3. Quantitative PCR

The competitive PCR was carried out as already described (Gilliland et al., 1990; Ferre, 1992). In a total volume of 40 μl DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four deoxynucleotide triphosphates (Pharmacia, Uppsala, Sweden), 0.5 μM of each forward and reverse primer and 2.5 U *Taq* DNA polymerase (Pharmacia) were added. The first step was for 1 min at 94°C, followed by 30 cycles of denaturation for 30 s at 92°C, annealing for 1 min (S672/S673: 59°C, S674/S675: 59°C, S700/S701: 53°C, S708/S709: 50°C) and extension for 2 min at 72°C (thermal cycler PTC-200, MJ Research, Watertown, USA). The PCR fragments were separated by electrophoresis through 1% agarose gels, visualised by UV light after ethidium bromide staining, documented and quantified. A videocamera and the software package of Cybertech (Cybertech DS1, Cybertech, Berlin, Germany) was used to determine the equivalence concentration where standard and target DNA concentration were identical. The competitor DNA was added at concentrations ranging from 5 ag to 500 fg. This corresponded to about 1.5 and 150 000 molecules.

2.4. Primer, target and competitor DNA

The plasmids, primers and fragment sizes obtained by PCR are listed in Table 1. The plasmids pJKS224, pJKS230 and pJKS219 were generated by amplification of fragments of *agp*, *cp*21 and *aphA* from transgenic beet DNA and inserted between the *Pvu*II sites of pUC18. The plasmids with the competitor DNA were generated by deleting a *Pvu*II fragment from pUC18

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Table 1
Target and competitor DNAs

Primer sequence	T_{anneal} (°C)	Target DNA		Competitor DNA	
		Plasmid/gene	Fragment size (bp)	Plasmid	Fragment size (bp)
S672: ATACGCAAACCGCCTCTCC S673: ATACCGCATCAGGCGCCAT	59	pUC18	434	pADI2.2	800
S700: TGGCAGAAGCACATTGA-CAC S701: TTGGGAGGCTGTTGTG-TAAG	53	*agp* (pJKS224)	776	pJKS227	600
S708: CCAGGGACTTCAGCAGGTG S709: CAGGAACCGCAGGAGTGGA	50	*cp*21 (pJKS230)	177	pJKS232	350
S674: CTCTGATGCCGCCGTGTTC S675: GCCCATTCGCCGCCAAGCT	59	*aphA* (pJKS219)	618	pJKS222	800

The used primers and the sizes of the PCR fragments after quantitative PCR are listed. The plasmids which contain the target PCR fragments are shown in brackets.

(pADI2.2), a *Eco*RV/*Nsi*I fragment from pJKS224 (pJKS227), a *Nsi*I/*Sca*I fragment from pJKS230 (pJKS232) and a *Pst*I/*Sph*I fragment from pJKS219 (pJKS222) and replacing them with *Hae*II fragments from bacteriophage λ. It was verified that the constructed internal standard (competitor) DNAs had comparable efficiencies of amplification as the appropriate pUC18-based target DNAs using the method described by Scadden et al. (1992). The 5 pg target and competitor DNA were independently analysed.

2.5. Hybridisation of DNA

Total genomic DNA was isolated and digested with restriction endonucleases. After electrophoresis, the DNA was transferred onto a nylon membrane (Immobilon P, Millipore, Eschborn, Germany) and hybridised with the cloned PCR fragments of the target DNA, labelled by using a non-radioactive DNA labelling and detection kit (Boehringer, Mannheim, Germany). Hybridisation was carried out at 68°C in hybridisation buffer as described by the manufacturer.

2.6. Immunological methods

Neomycin phosphotransferase and CP21 protein were detected by sandwich ELISAs using a biotin–streptavidin amplification system (5'Prime 3'Prime Inc., Boulder, USA). Absorbance values at 405 nm were read in a microplate reader (model 3550, Bio-Rad, Munich, Germany).

3. Results and discussion

3.1. DNA disappears from cossettes during extraction

The plant material used (about 25–30 kg of beets) was collected from different field trials and subjected to standard processing in a pilot plant and analysed. Conventional beets free of BNYVV (A) and conventional BNYVV-infected beets (B) served as controls. Beets of transgenic varieties (C) from BNYVV free areas were compared with the controls.

Genomic DNA from fresh sugar beet cossettes could be prepared by standard DNA extraction

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methods based on phenol extraction and ethanol precipitation (Section 2). However, DNA could not be detected in ethidium bromide (EtBr) stained agarose gels when this method was applied to post-extraction beet cossettes (pulp) or raw juice either. Southern blot analysis of these gels using a labelled cDNA of ADP-glucose pyrophosphorylase as a reference for genomic sequences of *B. vulgaris* cells and fragments from *aphA* or *cp*21 genes in case of transgenic beets again gave negative results (data not shown). Obviously, DNA disappeared during the process of juice extraction at 70°C for unknown reasons.

3.2. Nucleases from beet extracts degrade DNA in raw juice

When purified nucleic acid from fresh sugar beet cossettes was added to raw juice at 70°C, a quick degradation of DNA was observed by EtBr-stained agarose gel electrophoresis (Fig. 2A). This pointed towards the presence of DNA degrading activities, e.g. nucleases in the raw juice.

To corroborate this point, 250 μg ml^{-1} pUC18 DNA were added to fresh raw juice samples and incubated for the periods indicated in Fig. 2B. The amount of pUC18 DNA added by far exceeded the calculated amount of $\sim$10 μg ml^{-1} whole cellular DNA, assuming total lysis of all beet cells. Under these conditions, the added pUC18 DNA was shown to be degraded within minutes.

The rate of this DNA degradation could be shown to be temperature dependent (Fig. 2C) having low efficacy at 4°C, a slow degradation at 37°C but a high degradation activity at 70°C. Protein denaturation measures such as heating of raw juice at 95°C for 10 min or phenol extraction of raw juice resulted in the protection of added beet genomic DNA or pUC18 DNA from degradation (data not shown).

3.3. Degradation of the agp, aphA and cp21 DNA during the sugar recovery process steps as analysed by PCR

The *B. vulgaris* genomic DNA content both in raw juice and pulp (sugar beet cossettes after

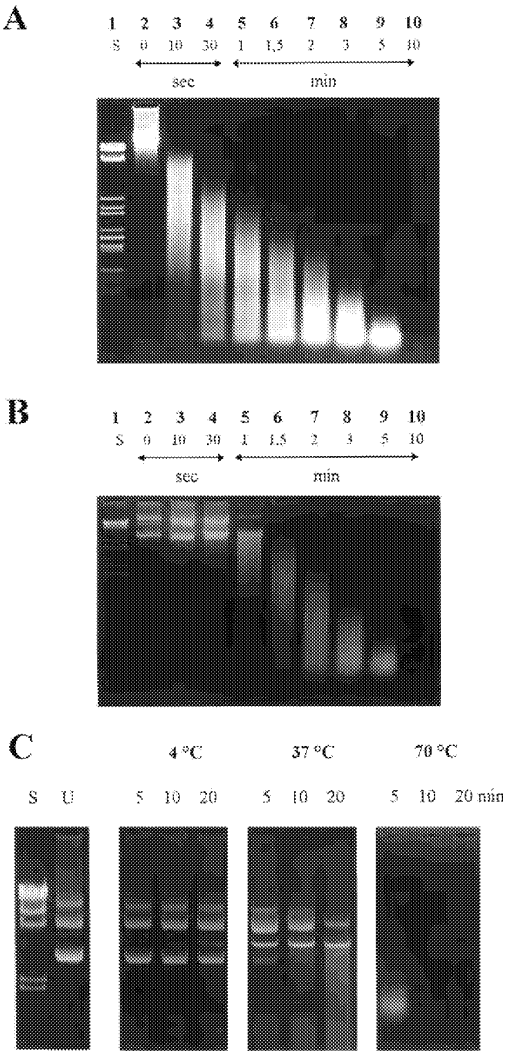

Fig. 2. Degradation of sugar beet chromosomal DNA (A), pUC18 DNA (B) and pUC18 DNA under various temperatures (C) in sugar beet raw juice. Chromosomal (A) or pUC18 DNA (B, C) were added to 500 μl raw juice from conventional beets free of BNYVV at a final concentration of 250 μg ml^{-1} at 70°C (A, B) or at 4, 37 and 70°C (C). Samples (20 μl), which were immediately extracted in the same volume of phenol/chloroform solution, were taken at the indicated times. Of the samples, 10 μl were separated by agarose gel electrophoresis; λ DNA cut with *Bgl*I (S) and uncut pUC18 DNA (U) were used as markers.

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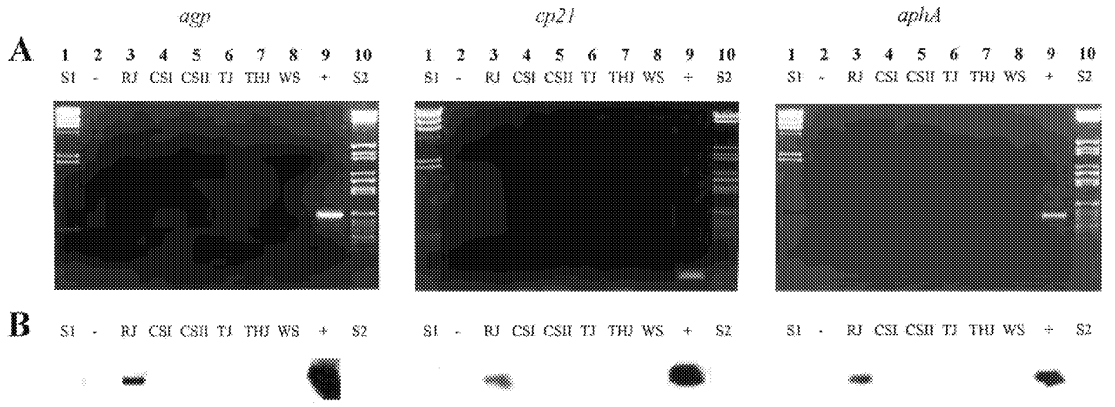

Fig. 3. Analysis of the *agp*, *cp*21 and *aphA* genes in raw juice (RJ), carbonatation sludge I (CSI), carbonatation sludge II (CSII), thin juice (TJII), thick juice (THJ) and white sugar (WS) from transgenic beets. The DNA was prepared as described in Section 2. DNA solutions (5 μl) were subjected to PCR using the primer set S700/S701 for the *agp* gene, S708/S709 for the *cp*21 gene and S674/S675 for the *aphA* gene; 100 pg of the appropriate plasmids containing the different target DNAs (pJKS224: *agp*, pJKS230: *cp*21 and pJKS219: *aphA*) were added as positive PCR controls (lane 9, +), the negative control was without DNA (lane 2, −). PCR reactions (10 μl) were separated via agarose gel electrophoresis (A), the DNA transferred to a nylon membrane and hybridised to DIG-labelled target DNA (B).

extraction of raw juice) was below detection limit of conventional methods such as Southern blot analysis. Therefore, the more sensitive PCR analysis was applied to these materials as well as to samples from the latter process steps.

Direct PCR analysis of raw juice samples with added pUC18 DNA gave only barely detectable signals, pointing towards factors in raw juice preventing efficient PCR amplification. Therefore, raw juice samples and those from subsequent processing steps were purified by phenol extraction followed by dialysis and DNA affinity chromatography. pUC18 DNA added to such purified samples could then be amplified as efficiently as the control with buffer (data not shown).

In samples from all processing steps, from raw juice to white sugar, from conventional as well as transgenic beets, DNA could not be detected using PCR with primers for *agp*, *aphA* and *cp*21 DNA (Section 2) followed by agarose gel electrophoresis and EtBr-staining (Fig. 3A). The more sensitive Southern blot hybridisation with digoxigenin-labelled DNA of the target DNAs gave clearly recognisable signals in PCR samples from raw juice only, but in none of the consecutive products. Chromosomal *agp* DNA was detected in raw juice from conventional and transgenic beets whereas the specific transgenic markers were found in raw juice from the respective beets only (Fig. 3B).

Quantification of DNA was performed by competitive PCR analysis according to Piatak et al. (1993). This comprises the comparison of the amounts of PCR products resulting from the co-amplification of a target sequence and an added internal standard of known concentration and recognisable by the same primer pair. Competitive plasmids for *cp*21, *aphA* and *agp* sequences as well as for pUC18 DNA were constructed (Section 2). The internal standard (competitor) DNAs were determined to have comparable amplification efficiencies as the appropriate pUC18-based target DNAs using the method described by Scadden et al. (1992).

The DNA content in raw juice being too low for proper quantification, it had to be concentrated 10-fold by DNA-affinity chromatography. Thereby, for each of the three gene fragments analysed equivalence concentrations of 2×10^4 molecules per 1 ml raw juice could be determined. This corresponds to about 5–10 fg of the constructed plasmids.

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Assuming a triploid genome (3 pg DNA per cell), a cell content of 10^6 cells in 1 g beet material (microscopically determined) and as 1 kg of sugar beets results in about 1.15 l of raw juice, this would mean a 100-fold reduction of the gene fragments (copy number basis). However, as the methodology is based on copy number comparison and the competitor DNA used is much smaller than chromosomes, the actual fragmentation of chromosomal DNA is to be expected to be much higher. The quantification of *agp* is shown as an example in Fig. 4.

3.4. DNA reduction potential of various sugar recovery process steps using added pUC18 DNA

The low number of DNA fragments detected in raw juice prompted us to validate all steps of the sugar recovery process for their potential to degrade or remove DNA.

For the first carbonatation step pUC18 DNA was added at a high dosage of 250 μg ml^{-1} to heat inactivated raw juice and liming and carbonatation was performed according to standard procedure. After filtration, samples of juice (so-called thin juice I) and sludge (sludge I) were retained and the main portion of juice subjected to a second liming and carbonatation treatment resulting in thin juice II and sludge II. The samples of thin juice I and II were dialysed and the DNA concentrated by affinity chromatography. Competitive PCR showed a 10^3-fold reduction of pUC18 DNA in the first and a 10^5-fold reduction in the second carbonatation step. Samples of sludge I and II were extracted, each with the same volume of water, dialysed and concentrated by affinity chromatography. They were shown by PCR to be free of DNA.

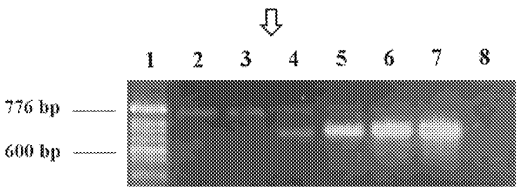

Fig. 4. Quantitative PCR of the *agp* DNA in raw juice: 15 μl of the 10 times concentrated and purified raw juice in the presence of 750 ag (lane 2), 5 fg (lane 3), 10 fg (lane 4), 30 fg (lane 5), 50 fg (lane 6) and 500 fg (lane 7) of competitor DNA pJKS227, respectively 220, 1470, 2940, 8800, 14 700 and 147 000 copies of pJKS227 were subjected to PCR. A negative control (lane 8) did not contain any DNA; 10 μl of the PCR reactions were separated via agarose gel electrophoresis and analysed as described in Section 2; λ DNA cut with *Bgl*I was used as molecular weight marker. The arrow indicates the equivalence concentration.

The results were verified by adding 0.250 μg ml^{-1} pUC18 DNA to heat inactivated raw juice. The competitive PCR confirmed a 10^3-fold reduction of pUC18 DNA in the first carbonatation step and showed this factor independent from the actual amount of DNA present. After the second carbonatation step no DNA was found, i.e. the DNA concentration was reduced by a factor of at least 10^5 in the second carbonatation. Again, there was no DNA to be detected in the sludge samples. In summary, during juice purification residual DNA fragments from raw juice will be reduced at least 10^8-fold.

The next step in the sugar recovery process is the multistep evaporation of thin juice II at a temperature range of 98–130°C and a residence time of $\sim$30 min to produce a thick juice. To simulate this step in the laboratory, a thin juice II sample with 250 μg ml^{-1} pUC18 DNA added was autoclaved at 121°C for 30 min. Thereby, a 10^3-fold reduction of pUC18 DNA concentration was shown by competitive PCR.

The last purification step in the sugar recovery process is crystallisation by evaporation of thick juice at a temperature of about 70°C followed by separation and washing of crystals in a sieve-basket centrifuge. This process step was carried out in the laboratory after adding 250 μg ml^{-1} pUC18 DNA to thick juice and evaporating to crystallisation. It was, however, not possible to wash the crystals in the laboratory centrifuge. Nevertheless, only about one-tenth of the DNA added could be found again.

The DNA degrading potential of nucleases in the raw juice was tested by adding pUC18 DNA (0.025 and 2.5 μg ml^{-1}) at 70°C. Samples taken at different times up to 120 min were analysed by competitive PCR. As shown in Fig. 5, pUC18 DNA was rapidly degraded within 15 min, reducing the copy numbers of intact target sequence by

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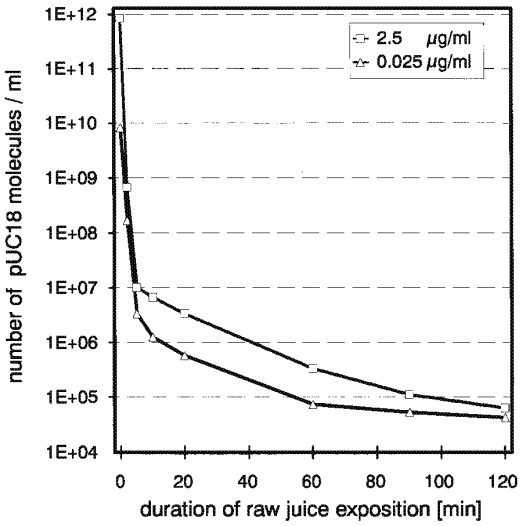

Fig. 5. Decrease of pUC18 DNA molecules in sugar beet raw juice. pUC18 DNA was added to 500 μl raw juice from conventional beets free of BNYVV at final concentrations of 0.025 (△) and 2.5 μg ml^{-1} (□); 20 μl samples were taken at the indicated times, immediately extracted with 20 μl phenol/chloroform and purified via a Qiagen column (Qiagen, Hilden, Germany). The amount of pUC18 molecules per μl raw juice was quantified via competitive PCR using the standard DNA pADI2.2.

a factor of about 10^5 (in the 2.5 μg ml^{-1} sample) followed by a slowing down of the degradation rate. This was found to be not due to inactivation of nucleases during the incubation period as a preincubation of raw juice for 120 min at 70°C led to similar degradation kinetics (not shown). It is assumed that the nuclease activity decreases at low DNA concentrations and increasing DNA fragmentation.

The factor of overall efficacy of DNA elimination under standard process conditions can be calculated to about 10^{14}. These activities include nucleolytic degradation in raw juice, irreversible adsorption on sludge, precipitation, denaturation and presumably hydrolysis due to alkaline pH and high temperature in the carbonatation steps, hydrolysis at the very high temperature during the evaporation step and exclusion of DNA from sugar crystals in the last step. The non-enzymatic steps should be independent of DNA concentrations and therefore capable of completely removing the low amounts of DNA left in the raw juice.

The reduction of biologically active DNA should even be greater as the DNA was considerably reduced in size in the raw juice and, later on, denatured to single-stranded DNA. This is because only small parts of the genes or pUC18 DNA were amplified and the actual size of the fragments may have even been smaller than the PCR fragments due to the extension of overlapping small fragments by *Taq* polymerase.

3.5. Proteins are removed during juice purification

The fate of the gene products of the transgenes was also looked at, e.g. neomycin phosphotransferase and BNYVV coat protein CP21. Applying ELISA methods for detection of neomycin phosphotransferase, 4×10^{-8} g ml^{-1} could be detected in raw juice from transgenic beets (C). Quantification of CP21 by the same technique showed that raw juice samples from BNYVV-infected conventional beets (B) contained 5×10^{-5} g ml^{-1} CP21, i.e. 10^3 times more than samples from BNYVV-free transgenic beets (C) which contained 3×10^{-8} g ml^{-1}. No AphA ($< 10^{-10}$ g ml^{-1}) or CP21 ($< 5 \times 10^{-9}$ g ml^{-1}) was found in pulp, thin juices, thick juice or white sugar from transgenic beets. This shows that proteins are efficiently removed during the juice purification steps.

In summary, extraction and purification steps of the standard sugar production process are very efficient in removal of nucleic acids and proteins irrespective of their origin. Consequently, the product, white sugar, is indistinguishable from its source: the transgenic beet varieties or conventionally bred controls.

References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., Beachy, R.N., 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232, 738–743.

Bevan, M.W., Flavell, R.B., Chilton, M.-D., 1983. A chimaeric antibiotic resistance marker gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

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Ehlers, U., Commandeur, U., Frank, R., Landsmann, J., Koenig, R., Burgermeister, W., 1991. Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots. Theor. Appl. Genet. 81, 777–782.

Ferre, F., 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl. 2, 1–9.

Gilliland, G., Perrin, S., Blanchard, K., Bunn, H. F., 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87, 2725–2729.

Hall, R.D., Riksen-Bruinsma, T., Weyens, G.J., Rosquin, I.J., Denys, P.N., Evans, I.J., Lathouwers, J.E., Lefèbvre, M.P., Dunwell, J.M., van Tunen, A., Krens, F.A., 1996. A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. Nature Biotech. 14, 1133–1138.

Herrera-Estrella, L., DeGreve, H., van Montagu, M., Schell, J., 1983. Expression of chimaeric genes transferred into plant cells using a Ti plasmid derived vector. Nature 303, 209–213.

Kallerhoff, J., Perez, P., Bouzoubaa, S., Ben Tahar, S., Perret, J., 1990. Beet necrotic yellow vein virus coat protein-mediated protection in sugar beet (*Beta vulgaris* L.) protoplasts. Plant Cell Rep. 9, 224–228.

Kieser, T., 1984. Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12, 19–36.

Piatak, M., Luk, K.-C., Williams, B., Lifson, J.D., 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques 14, 70–79.

Richards, K.E., Tamada, T., 1992. Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annu. Rev. Phytopathol. 30, 291–313.

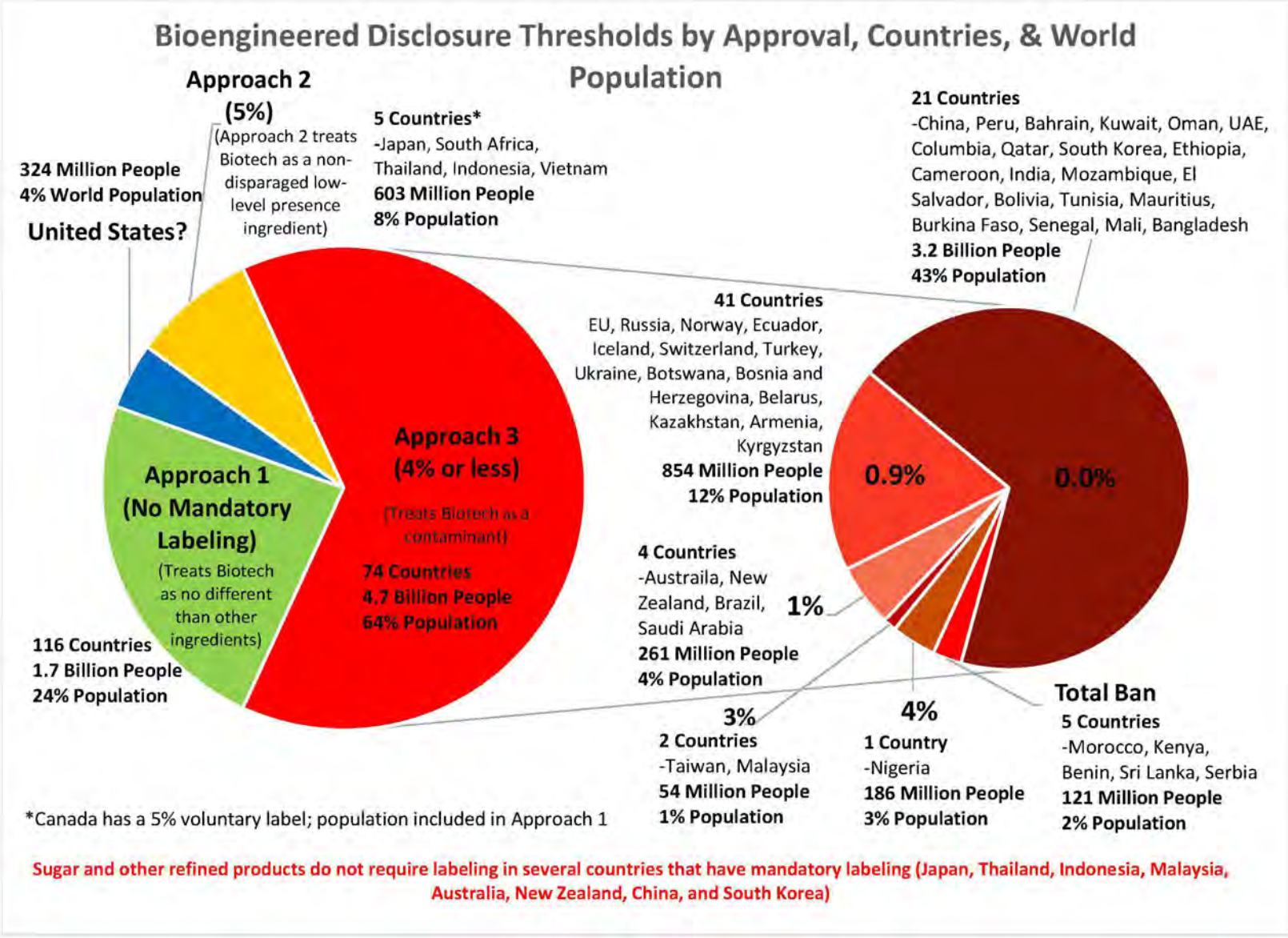
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scadden, D.T., Wang, Z., Groopman, J.E., 1992. Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. J. Infect. Dis. 165, 1119–1123.

Smith-White, B.J., Preiss, J., 1992. Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. J. Mol. Evol. 34, 449–464.

Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. Gene 33, 103–119.

ATTACHMENT 2


Bioengineered Disclosure Thresholds by Approval, Countries, & World Population
Approach 2 (5%)
(Approach 2 treats Biotech as a non-disparaged low-level presence ingredient)
5 Countries*
-Japan, South Africa, Thailand, Indonesia, Vietnam
603 Million People
8% Population
324 Million People
4% World Population
United States?
21 Countries
-China, Peru, Bahrain, Kuwait, Oman, UAE, Columbia, Qatar, South Korea, Ethiopia, Cameroon, India, Mozambique, El Salvador, Bolivia, Tunisia, Mauritius, Burkina Faso, Senegal, Mali, Bangladesh
3.2 Billion People
43% Population
41 Countries
EU, Russia, Norway, Ecuador, Iceland, Switzerland, Turkey, Ukraine, Botswana, Bosnia and Herzegovina, Belarus, Kazakhstan, Armenia, Kyrgyzstan
854 Million People
12% Population
Approach 3 (4% or less)
(Treats Biotech as a contaminant)
74 Countries
4.7 Billion People
64% Population
Approach 1 (No Mandatory Labeling)
(Treats Biotech as no different than other ingredients)
116 Countries
1.7 Billion People
24% Population
0.9%
0.0%
4 Countries
-Austraila, New Zealand, Brazil, Saudi Arabia
261 Million People
4% Population
1%
3%
2 Countries
-Taiwan, Malaysia
54 Million People
1% Population
4%
1 Country
-Nigeria
186 Million People
3% Population
Total Ban
5 Countries
-Morocco, Kenya, Benin, Sri Lanka, Serbia
121 Million People
2% Population
*Canada has a 5% voluntary label; population included in Approach 1
Sugar and other refined products do not require labeling in several countries that have mandatory labeling (Japan, Thailand, Indonesia, Malaysia, Australia, New Zealand, China, and South Korea)

MAJOR LABELING APPROACHES* *(Japan, Thailand, Indonesia, Malaysia, Australia, New Zealand, China, and South Korea do not require labeling of sugar and various other refined products)							
	Approach 1: **NO MANDATORY LABELING (116 Countries)**	**Approach 2:** **5% (5 Countries)**	**Approach 3:** **(4% - 3% - 1% - .9% - 0.0% - Bans)** **(74 Countries)**				
			4% **(1 Country)**	**3%** **(2 Countries)**	**1%** **(4 Countries)**	**.9%** **(41 Countries)**	**0.0%** **(21 Countries with 0.0%)** **(5 Countries with bans)**
ISSUES ⬇							
PRODUCTION, TRANSPORTATION, STORAGE COSTS (LOW, MEDIUM, HIGH)	Low	Medium/Low	Medium	Medium	High	High	High
IDENTITY PRESERVATION COSTS (LOW, MEDIUM, HIGH)	Low	Medium/Low	Medium	Medium	High	High	High
LIABILITY RISKS (LOW, MEDIUM, HIGH)	Low	Medium/Low	Medium	Medium	High	High	High
FOSTERS GE TECHNOLOGY (LOW, MEDIUM, HIGH)	High	Medium	Medium	Medium	Generally Low*	Low	Low
BIOTECH TREATED AS A "CONTAMINANT", NON-DISPARAGED "LOW-LEVEL PRESENCE" INGREDIENT, OR NO DIFFERENT THAN OTHER INGREDIENTS	Biotech treated as "normal" ingredient no different than others.	Non-Disparaged Low-Level Presence Ingredient	Mild Contaminant	Contaminant	Contaminant	Contaminant	Contaminant
Summary ➡	116 Countries (including our main trading partners, Canada and Mexico). Indicates support, trust, acceptance and fostering of biotechnology and biotech crop ingredients. Results in lower ingredients costs and consumer savings.	Japan, South Africa, Indonesia, Vietnam, and Thailand have 5% mandatory labeling thresholds. Canada and Hong Kong have 5% voluntary thresholds. The grain trade in Canada allows a 5% low level presence of biotech. This approach is the most supportive of biotech of the mandatory thresholds. The lowest cost approach and results in consumer savings. USDA Organic allows up to 5% non-organic ingredients. (Sugar and some other highly refined products are not required to be labeled in Japan, Thailand, and Indonesia)	Nigeria has mandatory labeling and draft legislation with a 4% threshold. The actual effects are unclear because the threshold is in draft form. In general, as biotech thresholds are less strict the associated costs go down.	Malaysia and Taiwan have a 3% threshold. This level generally results in lower prices for consumers and fosters the development of biotech. (Malaysia does not require labeling of highly refined products, including sugar).	Australia, New Zealand, Brazil, and Saudi Arabia have 1% thresholds. Australia and New Zealand (like the United States) don't require labeling if GE DNA is not present (highly refined foods such as sugars and oils). (Australia and New Zealand exempt sugar and other highly refined products from labeling)	The 28 EU Member States, Russia, Ecuador, Botswana, Bosnia and Herzegovina, Iceland, Norway, Switzerland, Turkey, Belarus, Kazakhstan, Armenia, Kyrgyzstan, and Ukraine have a .9% GE or GE-Derived Threshold. These countries generally shun GE crops and GE technology. This results in higher food costs to consumers. The thresholds are based on fear (precautionary principle) and not science. The current situation of the EU with very little cultivation of GE plants but high imports is not expected to change in the medium term. On July 3, 2016, Russia adopted FL 358-FZ, which prohibits the cultivation of genetically engineered (GE) plants. Regulations used as a non-tariff trade barrier to imports.	China is generally anti-biotech and as of December 30, 2016 had not approved any major food crops for cultivation or approved any GE food or feed crops developed by foreign biotechnology firms for domestic commercial production However, it is the world's largest importer of GE crops and one of the largest producers of GE cotton in the world. Government officials cite lack of public acceptance as an important factor behind the slow pace of biotechnology commercialization in China. Increases food costs. (Sugar and some other highly refined products are not required to be labeled in China and S. Korea)

COUNTRIES WITHIN LABELING APPROACH CATEGORIES

	The United States Government recognizes 195 countries. **116** countries don't have mandatory labeling requirements. Afghanistan Albania* (A candidate for admission into the EU and if accepted would adopt EU standards) Algeria Andorra Angola (No labeling laws but limits GE products to food aid) Antigua and Barbuda Argentina Azerbaijan The Bahamas Barbados Belize Bhutan Brunei Burma Burundi Cabo Verde Cambodia Canada Central African Republic Chad Chile Comoros Congo (Brazzaville) Congo (Kinshasa) Costa Rica Côte d'Ivoire Cuba Djibouti Dominica Dominican Republic Egypt Equatorial Guinea Eritrea Fiji Gabon Gambia Georgia Ghana Grenada Guatemala Guinea Guinea-Bissau Guyana Haiti Holy See Honduras Iran	Indonesia "Food registration procedures require a Genetically Modified Organism (GMO) or non-GMO statement for food containing potatoes, soybeans, corn, and their derivative products. This sometimes confuses BPOM officials when approving entry permits for these types of food. For example, BPOM regulations require that product derivatives which have undergone further refining **processes to the point where the GE material cannot be identified (to include but not limited to oils, fats, sucrose, and starch)** do not require any non-GMO statements. Japan (Eight crops – vegetables -fruits (soy, corn, potato, canola, cotton seed, alfalfa, beet, and papaya) and thirty-three processed foods that include more than 5% of these eight foods in weight are subject to labeling. The 5% tolerance applies only to GM varieties that have been approved in Japan." Beet sugar from GE sugarbeets is exempt from labeling. Other citation South Africa The Consumer Protection Act of 2011 has a 5% threshold but is on hold. Thailand Labeling: As for processed food containing GE plant materials, the Ministry of Public Health lists 22 food products which are subject to labeling requirements when the contents exceed the five percent tolerance threshold. Sugar is not included on the list. Vietnam On November 23, 2015, the government issued detailed guidance for the labeling of pre-packed GE foods with at least one GE ingredient having a content of **five percent** or higher of the total ingredients forming the product. Canada-(Voluntary Threshold)	Nigeria "Work in progress draft regulation stipulates products with four percent GE content to be labelled GM."	Taiwan **Not on the official country list of the US Government.** Taiwan Has a three percent GE threshold and expanded requirements to highly processed products which are primarily made of GE raw materials, such as oils and starches, where transgenic fragments or proteins may not be detected. Malaysia. In April 2013, Food Safety and Quality Division of the Ministry of Health (MOH) published new "Guidelines on Labeling of Foods and Food Ingredients Obtained through Modern Biotechnology." As of December 2016, it was still not implemented. Key elements 1) If the GE content is not more than three percent, labeling is not required, "provided that this presence is adventitious or technically unavoidable." 2) For single ingredient foods, the words "genetically modified (name of the ingredient)" must appear in the main display panel. 3) For multi-ingredient foods, the words "produced from genetically modified (name	Brazil Consumers must be informed when more than 1% of a product marketed as food for human or animal consumption contains or is produced from GMOs. Law passed in 2005. Australia/New Zealand "Exemptions from GM labelling: GM foods that do not contain any novel DNA or novel protein, and do not have an altered characteristic, do not require GM labelling. The decision not to label these foods was made because the composition and characteristics of these foods is exactly the same as the non-GM food. **These foods are typically highly refined foods, such as sugars and oils, where processing has removed the DNA and protein from the food, including novel DNA and novel protein.**"). Labelling is also not required when there is no more than 1% (per ingredient) of an approved GM food unintentionally present in a non-GM food. This means labelling is not required when a manufacturer genuinely orders non-GM ingredients but finds that up to 1% of an approved GM ingredient is accidentally mixed with the non-GM ingredient. GAIN Report Saudi Arabia If a product contains one or more GE plant ingredients with more than 1% GE content, the words (genetically modified) or (produced from genetically modified, name of the	EU (applies to all 28 member states)-**Not Cumulative** "genetically modified" or "produced from genetically modified [name of the organism]" must appear clearly next to the ingredient list. When GMOs are found in minute amounts in conventional food due to their adventitious or technically unavoidable presence during cultivation, harvest, or transport, the food is not subject to labeling provided that the amount present is less than 0.9%). Until the 1990's, the European Union (EU) was a leader in research and development of biotech plants. Under pressure from anti-biotech activists, EU and Member State (MS) authorities have developed a complex policy framework that has slowed down and limited research, development, and commercial production of biotech products. Due to repeated destruction of test plots by activists, programs are often limited to basic research inside laboratories and, in the past few years, several major private developers have moved their research operations to North America. Commercial cultivation of GE crops is minimal in the EU, as a result of strong regulatory constraints. The current situation of the EU with very little cultivation of GE plants but high imports is not expected to change in the medium term. Russia On July 3, 2016, Russia adopted FL 358-FZ, which prohibits the cultivation of genetically engineered (GE) plants and the breeding of genetically engineered animals in the territory of the Russian Federation. In addition, FL 358-FZ provides for stronger state monitoring and control of the processing and the importation of GE organisms and products derived from such organisms, and sets penalties for violations of this federal law. Products must be labeled if the presence of GE lines is over 0.9 percent. Journalists in Russia often report of consumer concerns with GE products. It is worth noting that labeling requirements increase the price of food containing GE ingredients. The price of examining products for the presence (or absence) of biotech components is high because the approved methods of testing are expensive. It is rare to find a "GMO" label in Russia. There currently is no ban on the registration of GE crops/lines/traits for imports for food and feed. However, Russia does not permit the importation of GE planting seeds. Therefore, U.S. exports of GE planting seeds to Russia are not allowed, and registration of GE lines in imports for processing into food and feed has become more and more difficult. Ecuador (contains or derived from) Botswana (No USDA citation available. Link to Botswana Investment & Trade Centre Information "You May Have to Show: Warnings, if they apply to your product: if the product contains GM ingredients, unless their presence is accidental and 0.9% or less " Bosnia and Herzegovina	China China's revised Food Safety Law, which entered into force on October 1, 2015, incorporates the existing regulations on biotechnology labeling into law (see GAIN Report CH15016). China's biotechnology labeling regulations, governed by MOA Decree 10 (see GAIN Report CH7053), require the labeling of approved agricultural biotech products, and prohibit the importation and sale of any unlabeled or mislabeled products. The 2015 Food Safety Law codifies into law existing biotechnology labeling regulations. The types of products subject to mandatory labeling include (list does not include sugar or cottonseed oil): 1. Soybean seeds, soybeans, soybean powder, soybean oil, and soybean meal 2. Corn seeds, corn, corn oil, and corn powder 3. Rapeseed for planting, rapeseeds, rapeseed oil, and rapeseed meal 4. Cottonseed 5. Tomato seed, fresh tomato, and tomato paste. **In September 2014, the government released remarks by President Xi Jinping affirming official support for biotechnology research, but calling for a cautious approach to commercialization. He also said that foreign companies should not be allowed to "dominate the agricultural biotechnology product market."** S. Korea Recently expanded their law. Expansion of mandatory biotech labeling to all detectable products (i.e. detectable biotech proteins): Under the previous Act, biotech labeling was required for products that contain detectable biotech component as one or more of the top five ingredients. However, the new Act requires biotech labeling for products that contain any detectable biotech Soy, corn, cotton, canola, sugar beet, and alfalfa and food products containing these crops are subject to biotech labeling requirement. The same requirement applies to both domestic and imported products. even for a minor ingredient. However, **exempts highly refined products such as cooking oil, sugar, soy sauce, etc. No supporting document is required to get exempted from biotech labeling requirements for the listed products. (allows 3% unintentional presence for unprocessed foods).** Ethiopia "Foods made with GE ingredients must carry a label with the following statement: 'genetically modified food'." Cameroon India On June 5, 2012, the government stipulated "every package containing genetically modified food shall bear at the top of its principal display panel the word

	Iraq Israel Jamaica Jordan* (Listed by some sources as having GE labeling laws but the USDA states they have not yet been adopted) Kiribati North Korea Kosovo* (A candidate for admission into the EU and if accepted would adopt EU standards) Laos Lebanon Lesotho Liberia Libya Liechtenstein Macedonia* (A candidate for admission into the EU and if accepted would adopt EU standards) Madagascar Malawi Maldives Marshall Islands Mauritania Mexico Micronesia Moldova Monaco Mongolia Montenegro* (A candidate for admission into the EU and if accepted would adopt EU standards) Namibia Nauru Nepal Nicaragua Niger Pakistan Palau Panama Papua New Guinea Paraguay Philippines Rwanda Saint Kitts and Nevis Saint Lucia Saint Vincent and the Grenadines Samoa San Marino Sao Tome and Principe Seychelles	**USDA Organic May contain, up to 5%: a. nonorganically produced agricultural ingredients which are not commercially available in organic form**		of the ingredient)" should appear in list of ingredients and "contains genetically modified ingredient" must be stated on the main display panel. 4) Highly refined foods, **defined as those where processing has removed all novel DNA and protein, are exempt from labeling requirements (refined oil, sugar, corn syrup, honey and dextrin).** 5) Meat from animals fed with GE grains do NOT need to be labeled. 6) Only GE crops that have been approved by NBB can be used for foods and food ingredients.	ingredients) shall appear clearly and easily to read in parentheses immediately following the ingredient(s) concerned, with same font size and different color. ("no retail packed food products with positive biotech labeling have been imported into the Kingdom to date. In general, Saudi importers of retail-packed food products do not import foods with GE content over 1 percent that requires labeling. They are concerned that biotech labeling could jeopardize their product image and result in losing market shares, since Saudi consumers have limited knowledge about agricultural biotechnology.")	Iceland Norway-(.9% for approved products and .5% for products that have not undergone risk assessment) Switzerland Turkey Belarus Kazakhstan Armenia Kyrgyzstan Ukraine Non-GMO Project "Preserving and building the non-GMO supply chain is a critical step of transitioning toward a safe, healthy food supply for future generations." Mission statement is to also "change the way our food is grown and made."	"GM." Industry sources report that there has been no enforcement of the labeling requirement by DCA. As the government is still in the process of establishing labeling regulations for GM foods, the future status of the DCA GM labeling regulation remains uncertain. Mozambique "Compulsory labeling of GE products or food containing GE ingredients is necessary based on the Mozambique Biosafety Legislation." El Salvador "Labeling for food products that contain GEs is required under Article 128 of the Consumer Law; however, this rule is currently not being enforced." Peru Has moratorium on planting of biotech crops. The moratorium includes three exceptions: 1) laboratory research; 2) use in pharmaceuticals and veterinary products; and 3) use in food, animal feed and in food processing. Mandates the labeling of GE content products Zero tolerance. Peru has yet to establish a threshold level of detection, nor has it clarified scientific and technical considerations for standards settings. Bolivia (no USDA citation available so link to Commerce) Colombia The MHSP issued regulatory Resolution 4254 establishing the requirements for labeling of food derived from modern biotechnology in 2012. The resolution requires labeling information for product health and safety, such as potential allergenicity. Labeling must also address the functionality of the food, as well as the identification of significant differences in the essential characteristics of the food. In addition to Resolution 4254, the Colombian government drafted a Technical Annex to supplement the Resolution, but the Annex is still in internal discussion within the MHSP. There remains no indication when the Annex will be finalized and published/notified. Tunisia "Tunisia's Ministries of Trade and of Public Health published a joint order on September 3, 2008 (Art. 7) calling for mandatory labeling of all GE food ingredients and products. However, this law is not clear on what types of products are covered or the percentage of GE material that is allowed. There is also no clear understanding of which entity is responsible for enforcement." Mauritius Bahrain Kuwait Oman

	Sierra Leone Singapore Solomon Islands Somalia South Sudan Sudan Suriname Swaziland Syria Tajikistan Tanzania Timor-Leste Togo Tonga Trinidad and Tobago Turkmenistan Tuvalu Uganda Uruguay Uzbekistan Vanuatu Venezuela Yemen Zambia Zimbabwe						Burkina Faso "The biosafety law requires that any GE product intended for distribution or marketing on the national territory must be packaged and labelled in an indelible and non-modified manner in order to ensure the protection of ethical and cultural values and to avoid any risks for the environment as well as human and animal health. Also, all GE product developed on the national territory shall be packaged and labelled by the producer or the dispatcher with the indication "Produced on the basis of genetically modified organisms" or "Containing genetically modified organisms" in conformity with complementary standards defined by the competent national authority in cooperation with other departments concerned. The terms of labelling are established on the basis of a decree adopted by the Council of Ministers. Oman" Senegal "The law states that all GE products used for direct animal or human food or for transformation or introduction into the environment should be labeled 'contains GMOs'." Mali "The law has provisions covering the import, export, transit, contained use, and release or introduction into the market of any GE products, be it for pharmaceutical, food feed or other agricultural proposes. There is also provision in the law for mandatory labeling for all products made from GE." United Arab Emirates Qatar Bangladesh **USDA Organic- From Policy Memo April 15, 2011 "Compliance with the organic standards entails that operations have verifiable practices in place to avoid contact with GMOs. Since organic certification is process-based, presence of detectable GMO residues alone does not necessarily constitute a violation of the regulation. The inadvertent presence of genetically modified material does not affect the status of the certified operation and does not result in loss of organic status for the organic product."** **TOTAL BANS (5)** Morocco **has a total GMO Ban:** Morocco neither produces nor allows importation of agricultural products derived from biotechnology for human consumption. Morocco's heavy reliance on the EU market as the principal destination for its agricultural exports has instilled a reluctance among policy makers and producers to

							accept biotechnology products. Morocco tolerates biotech products for use in its animal feed sector, but bans genetically engineered (GE) products for human consumption. Kenya On December 1, 2016, Kenya's National Assembly Agriculture committee recommended that the import ban on GE products be upheld until a new legislation on food safety of GE foods for human consumption is developed. Kenya does not commercially produce GE crops or GE seeds. No plants are registered for cultivation, import and export in Kenya. Benin "Although the government of Benin has ratified the Cartagena Protocol in March 2005 and established a National Biosafety Committee, if Benin still enforces a moratorium prohibiting the production, sale and import of biotech crops and foods. Serbia "Serbia strictly prohibits all imports, production, and commercial growing of genetically engineered (GE) crops or products containing GE traits. Sri Lanka According to the Ministry of Healthcare and Nutrition's Food (Control of Import, Labelling, and Sale of Genetically Modified Foods) Regulation 2006, Sri Lanka prohibits the import, sale, storage, and distribution of any genetically engineered (GE) or GE-derived products for human consumption. This includes any food item containing GE materials, or any food product which contains GE-derived ingredients."

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July 2, 2018

United States Department of Agriculture
Agricultural Marketing Service
Docket Clerk
1400 Independence Avenue, SW
Room 4543 - South
Washington, DC 20250

Submitted via www.regulations.gov

Re: Proposed Rule – National Bioengineered Food Disclosure Standard – Doc. No. AMS-TM-17-0050 (83 Fed. Reg. 19860 (May 4, 2018)).

Dear Sir/Madam:

The American Farm Bureau Federation (Farm Bureau) appreciates this opportunity to provide comment on the USDA Agricultural Marketing Service's (AMS) proposed rule to implement the National Bioengineered Food Disclosure Standard, Pub. L. 114-216, (the NBFDS or Act).

Farm Bureau is the country's largest general farm organization with nearly 6 million member families, and representing every type of crop and livestock production across all 50 states and Puerto Rico. To remain internationally competitive and lead the world in achieving the productivity and efficiency gains required to meet the food, fiber and fuel demands and environmental challenges of the 21st century, U.S. agriculture must stay on the cutting edge of technology.

With the use of bioengineered seeds, Farm Bureau's farm families produce safe food and raise healthier and more productive crops while providing a broad array of environmental benefits to help meet long-term sustainability objectives. We understand and support the consumer's desire to know what is in their food. However, our concerns have always been that any mandated disclosures must not disparage biotechnology, impose undue regulatory burdens or create market discrimination when there are no material differences between conventional foods and foods derived from biotechnology. We worked with Congress in drafting the NBFDS and supported it because it strikes the correct balance between transparency, accuracy and fairness. It prevents a state-by-state patchwork of food labeling requirements that would have driven up food costs for consumers.

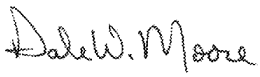
We applaud AMS for addressing stakeholders' competing views on the scope of the NBFDS by setting forth a number of options for the final rule. Our overriding concern, however, is that some of the options being considered, if adopted, have the potential to harm U.S. agriculture and stifle American farming innovation by presuming or implying that refined ingredients like sugars and oils, derived from a BE crop, contain genetic material when sound science shows they do not. Above all else, AMS must ensure that the NBFDS is a marketing standard, not a health, safety or nutritional standard. Congress expressly recognized that "the comprehensive federal

review process has determined that foods produced using bioengineering are safe and not materially different in any way from those made using other methods."[1]

As members of the Coalition for Safe Affordable Food we support many of the Coalition's comments and recommendations on the NBFDS. However, as referenced in the Coalition's comments, the members of the Coalition have diverging views on mandatory disclosure of refined ingredients, the BE food list, voluntary disclosure, and thresholds. In separate comments, with other national agricultural groups and state Farm Bureaus, we explain our unified and strongly held position on each of those issues. The comments that follow offer a more detailed explanation of our positions.

We appreciate your thoughtful consideration of our comments and will answer any questions or provide additional details should those be needed.

Sincerely,



Dale Moore
Vice President, Public Affairs

[1] S. Rep. No 114-403 (2016) at 2.

EXECUTIVE SUMMARY

In enacting the NBFDS, Congress expressly defined a bioengineered food (BE Food) as one that "contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques; and (B) for which the modification could not otherwise be obtained through conventional breeding or found in nature" and provided guiding principles for its implementation, which include that:

(1) the NBFDS not treat bioengineered food differently from its non-bioengineered counterpart;

(2) AMS "take every effort to minimize the impacts [of the NBFDS] on growers, handlers, processors, manufacturers, distributors, retailers and consumers;"[2]

(3) AMS minimize the impacts on all aspects of the domestic and international value chain;[3] and

(4) AMS provide "exemptions and other determinations under which a food is not considered a bioengineered food."[4]

Adhering to these principles, we discuss in detail below the following points and recommendations:

- **Definition of "Bioengineering" and "Bioengineered Food."** We support AMS's statement in the proposed rule that the "amended Act defines 'bioengineering' with respect to a food, as referring to a food '(A) that contains genetic material that has been modified through in vitro recombinant through in vitro recombinant deoxyribonucleic acid (DNA) techniques; and (B) for which the modification could not otherwise be obtained through conventional breeding or found in nature.' 7 U.S.C. 1639(1). In accordance with its statutory mandate and for purposes of consistency, AMS proposes to directly incorporate this statutory definition into the definition of 'bioengineered food' without further interpretation of what 'bioengineering' means."

- **AMS should not include highly refined ingredients in the definition of a BE Food (Position 1).**[5] Position 1 is supported by numerous scientific studies demonstrating the absence of genetic material from refined ingredients and AMS's own economic analysis showing that exempting refined sugars and oils from the definition of a BE Food would not reduce the number of foods subject to disclosure and would be far less costly than

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[2] Senate Report at 8.
[3] *Id.*
[4] *Id.*
[5] AMS continues to refer to processed sugars and oils as "highly refined ingredients." However, the more appropriate term is simply "refined ingredients." Highly processed or refined ingredients typically refer to multi-ingredient mixtures processed to the extent that they are no longer recognizable as their original plant/animal source, e.g., candy, tomato sauce, ice cream, etc. In contrast, when a single isolated food component, such as sugar, is obtained by extraction or purification using physical or chemical processes, it is typically referred to as "refined." *See e.g.,* Poti, J.M., et al., Is the degree of food processing and convenience linked with the quality of food purchased by US households, 101 *Am. J. Clin. Nutr.* 1251-1262 (June 2015). For these reasons, we recommend USDA to use the term "refined ingredients" when referring to single food components such as sugars and oils.

requiring product testing to prove the absence of genetic material.

- **If AMS is inclined to include refined products in the definition of a BE Food under Position 1, AMS must adopt the undetectable rDNA factor and condition.** Including refined products in the definition of a bioengineered food without providing a mechanism to exclude products that do not contain modified genetic material is contrary to Congress's express intent that the NBFDS apply only to foods that contain modified genetic material. It also treats refined ingredients derived from BE crops differently from foods derived from their non-bioengineered counterparts when they are molecularly identical. Disparate treatment of identical products has significant economic impacts on consumers, growers and the entire supply chain.

- **AMS's proposed list of BE Foods confuses BE Foods with crops and creates a presumption that foods "derived from" certain crops are BE Foods contrary to Congress's intent that a bioengineered food "contain genetic material that has been modified."** We understand and support AMS's objective to create an easily referenced list to facilitate compliance with the NBFDS. However, creating lists of highly adopted and not highly adopted BE Foods by reference to BE crops, which AMS proposes to serve as the "linchpin" for determining whether a regulated entity needs to disclose a BE Food, contradicts Congress's intent that a BE Food contain modified genetic material and renders Position 1 and the undetectable DNA factor and condition superfluous. Rather, AMS should adopt a BE ingredient list. Exhibit 2 of the Regulatory Impact Analysis (RIA), modified to reflect ingredients that are outside of the definition of BE under NBFDS (e.g., refined products, enzymes) is an easy to understand list that would facilitate compliance with the NBFDS without creating false presumptions or contravening the intent of the NBFDS that a BE Food is one that contains modified genetic material. Alternatively, AMS could use Table 5 from the RIA which lists the top 50 ingredients that would likely trigger disclosure, provided it eliminates from the list those products that are outside of the definition of BE Food under the NBFDS, (e.g., sugars, oils, or excluded ingredients like enzymes). Adopting a BE ingredient list is the preferred method for regulated entities to make disclosure decisions because most food manufacturers, especially small food manufacturers, do not know what crops many ingredients are derived from. The RIA itself supports this approach.

- **If AMS is inclined to address voluntary claims for foods that are not within the definition of a BE Food, AMS should not endorse specific on-package claims that ingredients are derived from or sourced from BE crops.** We support food manufacturers' desire to be transparent and disclose additional information concerning ingredients that are not BE Foods under the NBFDS. If AMS is inclined to create any safe harbors, which is not envisioned in the proposed rule, or provide guidance for such claims, endorsing on-package claims that ingredients are derived from or sourced from BE crops would create confusion because consumers would presume that "sourced or derived from" means the food is BE. Not only would this be misleading to consumers, it would defeat Congress's objective to achieve national uniformity in the labeling of BE Foods. Rather, if sourced or derived from claims are made, they should be provided through other means, such as an electronic or digital link, that allows complete and

truthful information to be provided without creating a secondary claim or disclosure that could mislead consumers into believing the food is BE when it is not.

- **AMS should adopt a 5 percent threshold that allows for the intentional use of small quantities of BE ingredients.** The threshold AMS establishes impacts how biotechnology is viewed by consumers and global trading partners. A 5 percent threshold supports biotechnology; appropriately balances disclosure, market dynamics, and international trade; and is consistent with other U.S. regulatory programs, including the USDA Organic Program which allows up to 5 percent of non-organically produced agricultural ingredients. A lower threshold, such as 0.9 percent, would be more aligned with the Non-GMO Project and European standards which denigrate biotechnology, stifle innovation and reduce choice for both farmers and consumers.

OVERARCHING PHILOSOPHY TO BE APPLIED TO THE RULE

Farm Bureau agrees that the focus of the final rule should be to establish a workable marketing standard, the NBFDS, for disclosure of BE information. Farm Bureau supports preserving the ability of food companies to voluntarily disclose information above and beyond what is required by the federal standard where that information is consistent with applicable federal law. Farm Bureau also agrees that the NBFDS should be a uniform national standard sufficient to ensure that federal preemption is maintained in accordance with AMS's statutory mandate.

Definitions

Refined Ingredients. Because use of the term "highly refined" in describing food ingredients and products is an inaccurate and poorly framed term in conjunction with the NBFDS, Farm Bureau recommends AMS instead use the more accurate term "refined," when describing ingredients and products.

Bioengineering. The statutory definition of the term "bioengineering" refers to a food "(A) that contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques; and (B) for which the modification could not otherwise be obtained through conventional breeding or found in nature."[6] The following recommendations are intended to clarify the use of certain key terms in the definition of "bioengineering."

Conventional Breeding. Farm Bureau supports inclusion of a definition to clarify "conventional breeding" in the final rule. Plant and animal breeding encompasses an evolving set of scientific disciplines and enabling methods that produce more effective breeding outcomes. Any discussion of breeding techniques that attempts to define or delimit "conventional breeding" should recognize this ongoing progression of breeding methods. What is considered "new" today may be deemed conventional or traditional in the future but does not result in fundamentally different breeding outcomes. This is consistent with the Act's bipartisan Senate Report that directed the NBFDS to "be technology neutral and reflect technological changes over time."[7] However, we recognize that there is value in providing clarification around the terms used in the statutory definition of "bioengineering".

AMS will need to clarify what is meant by a modification that "could not otherwise be obtained through conventional breeding." We recommend that AMS provide a definition for those modifications that could be obtained through conventional breeding together with a non-exhaustive set of examples of resulting modifications that describe an organism's potential genetic variability within its inherently diverse gene pool. AMS should avoid a static listing of current breeding techniques because any such list would ignore the constantly evolving science and hinder development of future enabling technologies that make the improvement of our food supply more efficient to accomplish. We also propose that AMS provide further guidance through explanatory text in the preamble to the final rule. Our suggested definition and explanatory text follow.

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[6] 7 U.S.C. 1639(1).
[7] S. Rep. No. 114-403 (2016) ("Senate Report"), at 4.

Suggested Definition:

"Modifications that could be obtained through conventional breeding" refers to a wide range of modifications obtained through methods that use an organism's potential genetic variability within its gene pool, such as, but not limited to, induced mutagenesis, somaclonal variation, induced haploidy, marker assisted breeding, and other methods that enable movement of existing genetic material between related organisms, such as, but not limited to, breeding crosses within the species, wide and bridging crosses, cell fusion, and embryo rescue.

Suggested Explanatory Text:

The goals of breeders have always been to create new variations of plant or animal characteristics, to provide solutions for diseases and pests, to increase tolerance to environmental stress, to improve quality and yields, and to meet consumer expectations. Breeding depends upon genetic variability within and across related species as a basis for developing new plant and animal varieties with improved traits. Breeders use this genetic variability to create new varieties through the movement of genetic material between different varieties within species, between closely related species, or closely related genera. They utilize a range of breeding methods in the process, such as wide crosses, bridging crosses, and embryo rescue. In vitro generated nucleic acids can be used to recreate or "mimic" many molecular changes or genetic variations that occur naturally or via conventional breeding. Plants and animals bred using these methods do not contain a transgenic insertion and, therefore, would not meet the definition of "bioengineered" and should be exempt from mandatory disclosure.

Regarding microbes, the concepts of "breeding techniques" and "conventional breeding" have limited applicability, especially with respect to methods for genetically modifying microbes that are food, that produce molecular substances added to food, or that carry out biological processes used in food production and processing. Over many decades, a wide array of methodologies, all derived from or based upon natural microbial methods of genetic modification, have been used to change the prokaryotic and eukaryotic microbes used in the manufacture of food and food ingredients. These methodologies are viewed as "conventional" because of their long history of safe use in many common foods. Over time, these methods have been altered and improved, and these improvements will continue as more is learned about microbial molecular genetics. Each of these methods should be considered "conventional breeding" under the Act and products resulting from these techniques would not be subject to mandatory disclosure.

Found in Nature. Farm Bureau recommends that AMS include a definition in the final rule and provide further guidance through explanatory text in the preamble to the final rule. Our suggested definition and explanatory text follow.

Suggested Definition:

"Found in nature" refers to the kinds of genetic modifications which can occur in nature within the genome of an organism, without human intervention.

Suggested Explanatory Text:

> Examples of such genetic modifications found in nature include, but are not limited to, deletions, insertions, substitutions, duplications, and translocations of genetic sequences within the organism's own genome. Changes can vary from single nucleotides to whole genes or larger segments of genetic material. Such modifications can occur through a variety of natural processes, including, but not limited to: crossing over in meiosis and sexual reproduction; microbial conjugation, transformation and transduction; transposon activity; horizontal gene transfer; and spontaneous gene mutations in somatic and germline cells.

In vitro recombinant DNA techniques can also be used to "mimic" the end points of various types of changes to genes that occur in nature, independent of human intervention including, but not limited to, crossing over in meiosis and sexual reproduction; microbial conjugation, transformation and transduction; transposon activity; horizontal gene transfer; and spontaneous gene mutations in somatic and germline cells. These are the types of techniques that would result in modifications that could "otherwise be found in nature" and the resulting food products would not be considered BE and would not be subject to disclosure under the NBFDS. When *in vitro* recombinant DNA techniques are used to create combinations of genetic elements that could not "otherwise be found in nature," food products containing these constructs would be considered BE and subject to mandatory disclosure under the NBFDS, unless otherwise excluded.

Farm Bureau strongly recommends that AMS not use an approach that would rely on intellectual property protections as a method in determining whether a modification could not otherwise be found in nature. As a threshold matter, whether a BE trait is patentable (i.e., is a natural product but not a product of nature) is a completely separate question from whether the trait could be found in nature. That said, even if the tests were analogous, there is much uncertainty in the state of patent law and biotechnology such that guidance from the U.S. Patent and Trademark Office on the issue would be of little help.

"Bioengineering" and "Bioengineered Food." Farm Bureau supports incorporation of the statutory definition of "bioengineering" into the final rule, as suggested by AMS in the proposed rule and, further, in accordance with USDA's statutory mandate and for purposes of consistency, Farm Bureau agrees with the proposal by AMS to directly incorporate the statutory definition of "bioengineering" into the definition of "bioengineered food" without further interpretation.

AMS SHOULD ADOPT POSITION 1 AS THE DEFINITION OF A BE FOOD.

The Preamble to the proposed rule discusses two competing views on whether refined foods should be included within the scope of the NBFDS and invites comment on three specific issues: (1) additional studies that address the presence of genetic material in refined foods, (2) the cost of implementation, including whether the scope of foods subject to the NBFDS would lower costs to affected entities, and (3) which position is the better interpretation of the statutory definition. We address each of these issues below to demonstrate that AMS should adopt Position 1 because it is grounded in science, does not impose unnecessary and unreasonable economic burdens on farmers, consumers, food manufacturers, supply chain distribution and

transportation systems, does not decrease the number of foods subject to the NBFDS, and is the better interpretation of the statutory definition of a BE Food.

1. The Peer-Reviewed Scientific Literature Establishes the Lack of Genetic Material in Refined Ingredients.

AMS correctly cites to a number of studies that demonstrate the absence of genetic material in refined sugar. These include a study conducted by German scientists which examined the fate of DNA and protein during the standard purification steps of the sugar extraction process from both conventional sugarbeets and sugarbeets genetically engineered with the coat protein CP21 to confer resistance to a certain virus.[8] This study is particularly important because it not only failed to detect DNA and protein beyond the early raw juice stage of the refining process, it estimated that the beet sugar clarification process had the potential to reduce the amount of sugarbeet DNA by a factor of ten to the fourteen (a hundred trillion or 0.00000000000001), which exceeds the total amount of DNA present in sugarbeets. AMS also cites to Oguchi, et al. (2009) that also found that sugarbeet plant DNA is degraded and removed early in the sugar extraction process and is therefore not present in the finished sugar.[9] Indeed, the Oguchi study was the basis upon which Japan exempted beet sugar from its mandatory GMO labeling requirements.[10] With respect to sugar produced from sugar cane, AMS correctly cites to Joyce, et al. (2013) and Taylor et al. (2009) demonstrating the absence of genetic material in refined cane sugar.[11] In addition, Pauli et al. (2000), did not find DNA in either raw or refined cane sugar.[12]

The science is further confirmed by a study published in March 2018.[13] Specifically, Brazilian researchers examined whether sugar produced from sugarcane genetically modified to express the Cry1Ab protein to control the sugarcane borer (*Diatraea saccharalis*) contained transgenic material. The study found that clarified juice, molasses, and raw sugar showed no detectable levels of Cry1Ab protein. Similarly, no heterologous DNA was detected in clarified juice and downstream products including raw sugar. As the researchers conclude, the results are in agreement with the results of other studies that investigated the degradation of specific DNA fragments inserted into genetically modified sugarcane (NptII) and glyphosate-resistant sugarbeet (CP4 EPSPS) that reported the complete elimination of the inserted DNA during

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[8] Klein, J., Altenbuchner, J., and Mattes, R., Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugarbeets. *J. of Biotechnology*, 60: 145-153 (1998).
[9] Oguchi, T., et al., Investigation of residual DNAs in Sugar from Sugar beet (*Beta vulgaruis L.*), *J. Food Hyg. Soc. Japan*, 50: 41-46 (2009), *available at* https://www.jstage.jst.go.jp/article/shokueishi/50/1/50_1_41/_pdf.
[10] In Japan, processed foods that contain detectable amounts of transgenic DNA or proteins must be labeled to indicate that genetically modified ingredients are used. Japan does not require sugar from transgenic sugarbeets to be labeled because the refined sugar does not contain transgenic DNA or proteins. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf.
[11] Joyce, P.A., Dinh, S-Q., Burns, E.M., and O'Shea, M.G. (2013), "Sugar from genetically modified sugar cane tracking transgenes, transgene products and compositional analysis," Proc. Int. Soc. Sugar Cane Technol." Vol. 28, pp 1-9; Joyce, P.A., Sedl, J.M. and Smith, G.R. (1999), "Laboratory crystallised sugar from genetically engineered sugar cane does not contain transgene DNA", Proc. Aust. Soc. Sugar Cane Technol., Vol. 21, pp. 502.
[12] Pauli *et al* (2000) Extraction and Amplification of DNA from 55 Foodstuffs. Mitt. Lebensm. Hyg. 91: 491-501.
[13] Cheavegatti-Gianotto, A., et al. "Lack of Detection of Bt Surgarcane CRY1Ab and NptII DNA and Proteins in Sugarcane Processing Products Including Raw Sugar (2018), Frontiers in Bioengineering and Biotechnology, Vo. 6, Art. 24 (2018).

processing to refined sugar.[14] Brazil, as the largest producer of cane sugar, relied on the Cheavegatti-Gianotto study to determine that sugar produced from genetically modified sugar cane is a "chemically defined pure substance" that does not fall within the scope of Brazil's Biosafety Law and therefore "is not a genetically modified organism or a derivative thereof."[15]

Importantly, the Brazilian study refutes any suggestion that the science is inconclusive about whether refined sugar contains genetic material. In the proposed rule, AMS cites Cullis et al. (2014)[16] as one study commenters claim shows that minute quantities of sugar cane DNA were detected in raw sugar (not for human consumption) after industrial milling prior to refining. Commenters do not understand the sugar refining process and misinterpret the scientific findings. Most importantly, as the Cullis study itself demonstrates, even if there is genetic material in the raw sugar, the refining process eliminates it altogether ("PCR failed to detect any sugar cane DNA in refined sugar."). [17] As Cullis concluded, the study's failure to detect DNA in the refined sugar is consistent with previous studies on the detection of DNA through the refining process[18].

Regarding refined ingredients as a class, Japan, Australia, New Zealand, Thailand, Indonesia, Malaysia, and South Korea have strict labeling regimes, but do not require the labeling of refined ingredients from BE crops because they do not contain transgenic DNA or protein. Indeed, Japan's labeling laws do not apply to corn oil, corn starch, dextrin, starch syrup, hydrolyzed protein derived from BE corn; soy sauce, soybean sprout, margarine, hydrolyzed protein derived from BE soy; canola oil derived from BE canola; or sugar derived from BE sugarbeets because they "do not contain traces of DNA."[19] Similarly, refined foods such as sugars and oils produced from BE crops are not included in Australia or New Zealand's mandatory GMO labeling laws because of the absence of DNA and protein in the refined product and "because the composition and characteristics of these foods are exactly the same as the non-GM food."[20]

Indonesia's food registration procedures require labeling for food containing genetically modified potatoes, soybeans, corn and their derivative products. However, product derivatives, which have undergone further refining processes to the point where the genetic material cannot be identified (to include but not limited to oils, fats, sucrose, and starch), do not require any GMO statements.[21] In Malaysia refined foods, defined as those where processing has removed all novel DNA and protein, are not included in the labeling requirements (refined oil, sugar, corn

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[14] Klein et al., 1998; Oguchi et al., 2009; Joyce et al., 2013.
[15] Cheavegatti-Gianotto et al., 2018.
[16] Cullis, C., Contento, A., Schell, M., DNA and Protein Analysis throughout the Industrial Refining Process of Sugar Cane. International Journal of Agricultural and Food Research, North America, 3, jul. 2014. Available at: https://www.sciencetarget.com/Journal/index.php/IJAFR/article/view/437.
[17] Cullis, et al. at 14.
[18] Klein et al., 1998; Oguchi et al., 2009; Joyce et al., 2013.
[19] See Gain Report, Japan https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf;*See* USDA GAIN Report No.
[20] See USDA GAIN Report, Australia New Zealand: https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Canberra_Australia_11-27-2017.pdf
[21] *See* USDA GAIN Report No. 1526, Indonesian National Biosafety Commission for Genetically Engineered Products, available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_7-14-2015.pdf.

syrup, honey and dextrin).[22] Finally, South Korea recently expanded their labeling law but still does not include several refined products.[23]

2. *There is No Rational Basis to Include Refined Ingredients in the Definition of a BE Food While Excluding Other Food Products and Ingredients that May Contain Genetic Material.*

AMS proposes to exclude from the definition of a BE Food incidental additives such as enzymes, which are BE. The Coalition for Safe Affordable Food is also requesting that (1) incidental additives, processing aids, secondary direct additives; (2) food derived from insects or microorganisms that grow or feed on a BE substrate, such as a BE crop or other substance; (3) enzymes; (4) ingredients derived via fermentation regardless of whether the microorganisms used in the fermentation are derived using rDNA technology, and (5) food products with medicinal or supplementary applications be excluded from the definition of a BE Food. Each of these proposed and requested exclusions are food products and ingredients that are likely to contain genetic material. While we do not object to these food products and ingredients being excluded from the definition of a BE Food, we are frustrated that there is a willingness to exclude certain foods and ingredients that contain some level of genetic material, albeit small, but an unwillingness to clarify that refined ingredients do not meet the definition of a BE Food when scientific evidence unequivocally demonstrates that refined ingredients contains no genetic material at all. Such disparate treatment is not rationally related to the purpose of the NBFDS. Nor is it scientifically or legally justified.

A. Including Refined Products in the Definition of a BE Food Imposes Unnecessary and Unreasonable Economic Burdens on Consumers, Food Manufacturers, Supply Chain Distribution and Transportation Systems, and the Agriculture Value Chain.

In its RIA, AMS analyzed three scenarios for the scope of the NBFDS: (Scope 1) all foods and dietary supplements that have been produced through BE (including highly refined oils, sugars, and high fructose corn syrup); (Scope 2) exclusion of sugars and oils; and (Scope 3) exclusion of foods where the genetic material cannot be detected. As we understand it, Scope 2 equates to Position 1 described in the Preamble, Scope 1 equates to Position 2 without the adoption of the undetectable DNA factor and condition, and Scope 3 applies the proposed factor and condition undetectable DNA.

The RIA demonstrates that Position 1/Scope 2 (excluding refined products) does not result in fewer food products being subject to the NBFDS, nor does it impose unreasonable costs. However, the RIA's conclusion that the costs of Position 2/Scope 1 are the same as Position 1/Scope 2 does not consider all costs "stretching back to the farm" that would be incurred if refined products were presumed to be a BE Food. We show below that creating any presumption that refined ingredients are a BE Food results in product deselection and price differentials.

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[22] See USDA GAIN Report, Indonesia: https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_11-20-2017.pdf

[23] See USDA GAIN Report No. KS1716, Korea's New Biotech Labeling Requirements, available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Update%20to%20Korea's%20New%20Biotech%20Labeling%20Requirements_Seoul_Korea%20-%20Republic%20of_6-23-2017.pdf.

When these costs are considered, Position 1/Scope 2 (excluding refined products) is the lowest cost option. With respect to Scope 3 (the undetectable DNA factor and condition), the RIA confirms that it results in far fewer products being subject to the NBFDS and imposes far higher testing costs on the industry. For this reason alone, AMS should adopt Position 1 over Position 2 with the undetectable DNA factor and condition.

1. Clarifying that Refined Products do not meet the Definition of a BE Food Does Not Decrease the Number of Products Subject to the NBFDS.

One of the principal arguments that has been raised in opposition to clarifying that refined products do not meet the definition of a BE Food is that it would significantly decrease the number of foods subject to the NBFDS. Some have even suggested there could be 80 percent fewer products labeled as a BE Food. The RIA squarely refutes these claims.

As the RIA explains, the concept of nesting recognizes that most foods subject to the NBFDS are multi-ingredient foods, any one of which could potentially trigger disclosure under the NBFDS. The RIA therefore evaluated the number of food labels potentially subject to the NBFDS with and without refined sugars and oils included.[24] The RIA found that not including sugars and oils did not result in any noticeable difference in the number of labeled products subject to the NBFDS.[25] The RIA further found that dietary supplements are even less sensitive to the exclusion of refined oils and sugars, with only 0.5 percent of products required to be labeled under Scope 1 would be excluded under Scope 2. In other words, refined sugars and oils are not the ingredients that drive disclosure.

In contrast, the RIA demonstrates that adopting the undetectable DNA factor and condition, which would apply to many more foods than just refined foods, results in only 45 percent of labels being be subject to the NBFDS. Indeed, Exhibit 2 of the RIA demonstrates that only 28 ingredients would be exempt under Position 1/Scope 2, while 98 ingredients would be exempt under Scope 3 (undetectable DNA).

Excluding refined sugars and oils under Position 1 has no meaningful effect on the number of food labels subject to the NBFDS and therefore should not be a determining factor in AMS choosing Position 2 over Position 1. Adopting the undetectable DNA factor significantly reduces the number of food labels subject to the NBFDS and, as discussed below, imposes unnecessary costs.

2. The RIA Does Not Address the Market and Agricultural Impacts that Flow from Presuming Refined Ingredients are BE Foods Under Position 2/Scope 1.

The legislative history of the NBFDS makes clear that "the Secretary, when determining the amounts of a bioengineered substance that may be present in food, or the threshold requirement, shall *minimize the impacts on all aspects of the domestic and international value chain*," as well as "minimize the impacts on growers, handlers, processors, manufacturers, distributors, retailers, and consumers.[26] Moreover, the NBFDS "is not intended to increase the costs of food

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[24] RIA at 51.
[25] *Id.* (finding that under Scope 1, 66 percent of labels would be subject to the NBFDS and under Scope 2, 64 percent of food labels would potentially be subject to the NBFDS).
[26] Senate Report at 4, 8.

manufacturing or changes in distribution or handling."[27] Congress's intent that the NBFDS not disrupt domestic and international supply chains is reinforced by E.O. 13777, which established a federal policy to alleviate unnecessary regulatory burdens. Creating any presumption that refined ingredients are BE Foods when they not contain modified genetic material exacerbates impacts on growers, handlers, processors and the domestic and international value chain.

(a) The RIA fails to consider price impacts of presuming refined ingredients that do not contain modified genetic material are BE Foods under Position 2 when they are identical to all other refined ingredients from conventional crops.

The RIA requests comment on the potential market reaction to the NBFDS and in particular, solicits evidence of market reaction to products presumed to be BE Foods.

Farm Bureau recommends AMS adopt Position 1 and exclude refined ingredients from the definition of BE Foods to avoid market discrimination that results in higher consumer prices and harmful impacts to the agriculture value chain.

(b) Presuming Refined Ingredients are BE Foods harms the American farmer.

Disruption in the supply chain and disparagement of the technology harms the American farmer because demand for BE crops will decline, even though they improve crop yields and are more environmentally sustainable than conventional crops.[28] Indeed, when the Vermont law was enacted many farmers faced uncertainty regarding the future viability of their BE crops which have enabled farmers to adopt production practices that have significantly offset rising costs. These include increases in diesel prices,[29] land costs,[30] water costs,[31] industrial energy supplies,[32] seed, fertilizers and pesticides.[33]

If AMS creates any presumption that refined ingredients are BE Foods, the costs stretching back to the farm will be far greater than the RIA estimates. Farmers would have to begin producing non-BE crops. All the cost savings BE crops provide would be lost, and the cost to begin producing non-BE crops would be much higher. In other words, they would be cost prohibitive.

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[27] Id. at 7.

[28] "Crop biotechnology has contributed to significantly reducing the release of greenhouse gas emissions from agricultural practices. This results from less fuel use and additional soil carbon storage from reduced tillage with GM crops. In 2012, this was equivalent to removing 27 billion kg of carbon dioxide from the atmosphere or equal to removing 11.9 million cars from the road for one year." GM crops: global socio-economic and environmental impacts 1996-2012. PG Economics Ltd, UK, http://www.pgeconomics.co.uk/page/36/-gm-crop-use-continues-to-benefit-the-environment-and-farmers.

[29] US Energy Information Administration, "US Retail Diesel Prices," available at https://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=EMD_EPD2D_PTE_NUS_DPG&f=M.

[30] The price of land has increased from a national average of $1,830/acre to $3080/acre. *See* USDA, National Agricultural Statistical Service, "Land Values, 2017 Summary," (Aug. 2017), available at https://www.usda.gov/nass/PUBS/TODAYRPT/land0817.pdf.

[31] OECD, "Agricultural Water Pricing: United States," (2010) available at https://www.oecd.org/unitedstates/45016437.pdf.

[32] US Energy Information Administration, "Electric Power Monthly," (March 2018) available at https://www.eia.gov/electricity/monthly/epm_table_grapher.php?t=epmt_5_3.

[33] Univ. of Illinois, "Growth Rates of Fertilizer, Pesticide, and Seed Costs over Time," (July 2016) available at http://farmdocdaily.illinois.edu/2016/07/growth-rates-of-fertilizer-pesticide-seed-costs.html.

This could cause farmers to seek other crop alternatives, which could lead to major disruptions in domestic commodity supplies.

Congress instructed AMS to make "every effort . . . to ensure that farmers access to seed technology and not limit the options available to agricultural production" and directed USDA "to take every effort to minimize the impacts on growers."[34] Adopting Position 2 creates a presumption that refined ingredients derived from BE crops are BE Foods which is difficult to overcome in the market even if AMS also adopts the undetectable DNA factor and condition. AMS's proposed list of BE Foods exasperates the presumption and harms the industry. The risks to the American farmer are too significant for AMS to ignore science and adopt Position 2.

Impacting the American farmer contradicts E.O. 13790, which established an Interagency Task Force on Agriculture and Rural Prosperity (Task Force) to "identify legislative, regulatory, and policy changes to promote in rural America agriculture, economic development, job growth, infrastructure improvements, technological innovation, energy security, and quality of life."[35] In its first report, the Task Force expressly identified technological innovation as one key indicator of rural prosperity. Specifically, with respect to biotechnology, the Task Force noted:

> Biotechnology is another area of U.S. leadership, being a sector that has driven innovation in fuels, chemicals, manufacturing, and agriculture. In 2016, biotech crops were grown on over 170 million acres in the United States, including over 92% of corn, soybean and cotton total acreage, according to the Department of Agriculture's National Agricultural Statistics Service. Globally, the biotechnology sector is a driver of the "fourth industrial revolution," and presents and an incredible opportunity for American farmers and rural communities to thrive at the forefront of innovation.[36]

Any mandate that refined foods that do not contain genetic material be subject to the NBFDS undermines the advancement of technology for agricultural production in direct contravention of E.O. 13790. It also perpetuates the misinformation that activists have used for decades to distort the truth about biotechnology, instilling fear in the general public when the global scientific community has repeatedly attested to its safety.[37] Indeed, in making clear that the NBFDS is a marketing standard, not a health, safety or nutritional standard, Congress recognized that "the comprehensive federal regulatory review process has determined that foods produced using bioengineering are safe and not materially different in any way from those made using other methods."[38] If there were any safety concerns, FDA, not USDA, would act under its authority.

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[34] Senate Report at 7.
[35] See Executive Order 13790, "Promoting Agriculture and Rural Prosperity in America" https://www.federalregister.gov/documents/2017/04/28/2017-08818/promoting-agriculture-and-rural-prosperity-in-america.
[36] Report to the President of the United States from the Task Force on Agriculture and Rural Prosperity (Oct. 2017), available at https://www.usda.gov/sites/default/files/documents/rural-prosperity-report.pdf.
[37] *See e.g.*, National Academy of Sciences, The Royal Society of Medicine, WHO, OECD, the American Medical Association, Food and Agriculture Organization of the United States, American Diabetes Association, and the Society of Toxicology.
[38] Senate Report at 4.

B. Position 1 is the Better Interpretation of the Statutory Definition of a BE Food.

Agency interpretations of statutes they implement are generally considered under the two-part inquiry articulated in *Chevron U.S.A., Inc. v. NRDC*, 467 U.S. 837 (1984). First, if Congress has "directly spoken" to the question at issue," the unambiguous intent of Congress controls.[39] If the statute is "'silent or ambiguous with respect to the specific issue," the agency's interpretation is given deference if it is reasonable.[40] Here, Congress unambiguously defined a BE Food as a "food that contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques."[41] Congress thoughtfully, deliberately and intentionally did not extend the scope of the Act to include ingredients derived from bioengineered crops.

The legislative history reinforces the plain language of the statute and makes clear that the definition of a BE Food set forth in the statute establishes the scope of the disclosure standard:

> "The Secretary of Agriculture is directed to establish a mandatory uniform national disclosure standard for human food that is or may be bioengineered. For this purpose, *the definition of bioengineering is set in statute and establishes the scope of the disclosure standard.* Congress intends an item of food to be subject to the definition if it contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and this same modification could not be otherwise obtained through conventional plant breeding or found in nature."[42]

Refined foods that do not contain genetic material do not meet the statutory definition of a BE Food. As demonstrated by the science discussed above, refined ingredients do not contain genetic material and therefore cannot be a BE Food within the scope of the NBFDS.

Some groups argue that Congress defined "bioengineering" in § 291(1) of the Act and gave the Secretary discretion in § 293(a) to define a BE Food. They say this reading of the Act is consistent with floor statements made by Members of Congress during debate and with a memo from USDA's General Counsel, which some incorrectly describe as a legal opinion. These groups are reading the statements and the memo out of context. Nevertheless, they cannot supplant the plain language of the NBFDS.

There is no provision in the NBFDS where Congress gave the USDA Secretary the discretion to rewrite the definition of a BE Food from a food that itself contains genetic material to any food derived from BE, a definition Congress expressly rejected. Position 2 modifies the statutory definition of a BE Food by creating a presumption that refined products are BE Foods because they are derived from BE crops. AMS's proposed lists of highly adopted and not highly adopted foods amplifies the presumption and further contravenes the statutory definition of a BE Food. The presumption also renders superfluous Congress's direction that the USDA Secretary

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[39] *Pharm. Research & Mfrs. of Am. v. Thompson*, 251 F.3d 219, 224 (D.C. Cir. 2001).
[40] *Citizens Coal Council v. Norton*, 330 F.3d 478, 481 (D.C. Cir. 2003) (*quoting Chevron,* 467 U.S. at 843).
[41] 7 U.S.C.§1639(1)(A).
[42] Senate Report at 3.

"determine the *amounts* of a bioengineered substance" that may be present in food to be considered a BE Food because it creates a zero threshold. As the Supreme Court has repeatedly made clear the "plain language" of a statute is the "'primary guide'" to Congress' preferred policy."[43] Here, the plain language makes clear that "bioengineering . . . *with respect to a food, refers to a food* . . . that *contains genetic material.*"[44]

Even if the definition of a BE Food were considered ambiguous, which it is not, adopting Position 2 would be an unreasonable interpretation of the NBFDS for four reasons. First, it signals to the market that refined products produced from BE crops are somehow different or less desirable than refined ingredients produced from non-BE crops contrary to Congress's direction that the NBFDS not treat BE Food differently from its non-BE counterpart. As discussed in section I -B above, this leads to price differentials and harmful market impacts.[45] Second, it creates chaos in the domestic and international supply chain contrary to Congress's direction that AMS minimize the impacts on all aspects of the domestic and international value chain. Third, there is no reasonable rationale for exempting from the definition of a BE Food foods that contain genetic material, such as incidental additives, enzymes, yeasts, and other BE ingredients but include in the definition refined ingredients that contain no genetic material whatsoever. Finally, adopting Position 2 and making refined products subject to the mandatory disclosure requirement compels commercial speech that is not truthful.[46]

I. IF AMS IS INCLINED TO INCLUDE HIGHLY REFINED PRODUCTS IN THE DEFINITION OF A BE FOOD UNDER POSITION 2, AMS MUST ADOPT THE UNDETECTABLE DNA FACTOR AND CONDITION.

If, despite the strong scientific evidence and international precedent that refined ingredients do not contain modified genetic material, AMS is inclined to adopt Position 2, then AMS must also adopt the undetectable rDNA factor and condition and make clear at the time the Final rule is published that refined ingredients do not meet the definition of BE Foods under the undetectable rDNA factor and condition. Including refined ingredients, in the definition of a BE Food without providing a mechanism to exclude them from the definition of a BE food is contrary to Congress's express intent that the NBFDS apply only to foods that contain modified genetic material. It also discriminates against refined foods like sugars and oils by treating them differently from their non-BE counterparts when the foods are molecularly identical, which leads to the harmful market impacts.

Including refined ingredients in the definition of a BE Food, but allowing their exclusion under the undetectable rDNA factor and condition is confusing and not necessary when the agency has before it multiple scientific studies demonstrating the absence of any genetic material in refined ingredients. It sends misleading messages to consumers by creating a presumption that refined ingredients are BE Foods but are excluded from the mandatory disclosure requirements. It also places an onerous burden on the industry to overcome the presumption, educate consumers on

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[43] *Sandoz, Inc. v. Amgen, Inc.,* 137 S. Ct. 1664, 1678 (2017) (quoting *McFarland v. Scott*, 512 U.S. 849, 865 (1994).
[44] § 291(1).
[45] *See Motor Vehicle Mfrs. Ass'n of U.S. v. State Farm Mut. Auto. Ins. Co.,* 463 U.S. 29, 43 (1983) (an agency's decision is arbitrary or capricious if it runs counter to the evidence before the agency, relies on factors which Congress did not intend, and/or is not otherwise the product of reasoned decision making).
[46] *See Zauderer v. Office of Disciplinary Counsel*, 471 U.S. 626 (1985) (First Amendment protects commercial speech and protects advertisers from compelled speech).

the benefits of BE crops, and gain consumer acceptance of the technology. "A Fresh Look", which is supported by Farm Bureau, brings farmers from across the country together to educate consumers about the benefits of GMO farming methods, including how BE crops allow farmers to produce food with less water, land, energy and pesticides.[47] A Fresh Look strives to, among other things, promote food marketing practices that address science-based health and environmental benefits — not spread misinformation to justify inflating prices for some foods, while playing on consumer fears to stigmatize other, equally healthy options. AMS should support these efforts, not create misleading presumptions that undermine them.

Finally, AMS notes that it may consider compatibility of the undetectable rDNA factor and condition with U.S. trading partners. However, we believe that Position 1 (excluding refined products from the definition of a BE Food) is more compatible with U.S. trading partners than creating a presumption that refined ingredients are BE Foods but are excluded from mandatory disclosure under the undetectable rDNA factor and condition. We are not aware of any country that requires industry to demonstrate through testing that its food products do not contain genetic material. Rather, countries have relied on published studies to themselves conclude that certain refined products are outside the scope of their mandatory labeling laws. For example, Japan relied on Oguchi, et al. (2009) to exempt beet sugar from its mandatory GMO labeling requirements[48] and Brazil relied on Cheavegatti-Gianotto, et al. (2018)[49] to determine that bioengineered sugar cane is a "chemically defined pure substance" that does not fall within the scope of Brazil's Biosafety Law and therefore "is not a genetically modified organism or a derivative thereof." We recommend AMS to do the same with respect to refined ingredients. There is simply no justification for creating a false presumption that refined ingredients are BE Foods but are not subject to mandatory labeling requirements when the agency has before it conclusive scientific evidence that refined ingredients are not BE Foods within the meaning of the NBFDS.

II. AMS'S PROPOSED LIST OF BE FOODS CONFUSES BE FOODS AND CROPS AND CREATES A PRESUMPTION THAT FOODS "DERIVED FROM" CERTAIN CROPS ARE BE FOODS CONTRARY TO CONGRESS'S INTENT THAT A BE FOOD "CONTAIN MODIFIED GENETIC MATERIAL."

AMS proposes to create two lists of BE Foods, one for "highly adopted" BE Foods and the other for "not highly adopted" foods. AMS proposes that these lists "would serve as the linchpin in determining whether a regulated entity would need to disclose a BE Food under the NBFDS." However, the BE Food lists are lists of BE crops - not BE Foods. By creating a list of BE crops to serve as the "linchpin" for determining whether disclosure is required makes superfluous any exclusion AMS provides for refined products under Position 1 or under the undetectable DNA

[47] For more information about A Fresh Look, *see* https://afreshlook.org/.
[48] In Japan, processed foods that contain detectable amounts of transgenic DNA or proteins must be labeled to indicate that genetically modified ingredients are used. Japan does not require sugar from transgenic sugarbeets s to be labeled because the refined sugar does not contain transgenic DNA or proteins. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf.
[49] Cheavegatti-Gianotto, A., et al. "Lack of Detection of Bt Surgarcane CRY1Ab and Nptll DNA and Proteins in Sugarcane Processing Products Including Raw Sugar (2018), Frontiers in Bioengineering and Biotechnology, Vo. 6, Art. 24 (2018).

factor and condition. Regulated entities will rely on the crop list, not the exclusions under within the law to make disclosure decisions. Thus, by default, AMS is defining a BE Food as one derived from a BE crop in direct contravention of the NBFDS.

A. AMS Should Create an Ingredient List to Facilitate Compliance with the NBFDS.

We support AMS's intent to facilitate compliance with the NBFDS. However, we recommend the creation of a BE ingredient list, which the RIA has already created through an extensive analysis of food product labels. Exhibit 2 of the RIA, modified to reflect ingredients excluded from the scope of the NBFDS, *i.e.*, refined products, enzymes, is an easy to understand list that would facilitate compliance with the NBFDS without creating false presumptions or contravening the intent of the NBFDS that a BE Food is one that contains genetic material. Alternatively, AMS could use Table 5 from the RIA which lists the top 50 ingredients that would likely trigger disclosure, provided it eliminates from the list those products excluded from the definition of a BE Food (e.g., sugars, oils, enzymes). Adopting a BE ingredient list is the preferred method for regulated entities to make disclosure decisions because most food manufacturers, and especially small food manufacturers, do not know what crops many ingredients are derived from. The RIA itself supports this approach:

> If the USDA provided a definitive list of final ingredients by type of disclosure (may contain, does contain), manufacturers' analysis would consist of matching their list of ingredients to the list of required disclosures. That would move most, if not all, products into the low cost category. Therefore, all else held equal, the more clarity USDA provides on which ingredients should apply each label type, the higher the potential savings.[50]

The attached document demonstrates a BE ingredient list is workable. The list is based on RIA Exhibit 2, not including refined ingredients or enzymes.

B. If AMS is Inclined to Create a BE Food List that Includes Bioengineered Crops, AMS Must Also Create an Excluded Ingredient List When the Final Rule Is Published.

Although its not the best approach for complying with the NBFDS, if AMS adheres to its proposal that the BE Food list reference BE crops, then we support the Coalition for Safe Affordable Food's recommendation that AMS also create an Excluded Ingredients List that identifies those ingredients that are excluded or not under the scope on the NBFDS either under Position 1 or the undetectable rDNA factor and condition. Providing an Excluded Ingredients List is the only way AMS can mitigate the false and misleading presumptions created by a crop list alone. However, because AMS has before it ample evidence that refined ingredients do not meet the statutory definition of a BE Food, it is imperative that an initial Excluded Ingredients List be published with the Final rule and that initial list include refined ingredients. If there is any delay between the publication of the Final rule and the creation of an Excluded Ingredient List, AMS will create confusion in the market and impose an onerous burden on producers of refined ingredients to overcome the false and misleading presumption that refined ingredients are BE Foods. We recommend that AMS create a BE Food list of ingredients, not crops.

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[50] RIA at 29.

III. IF AMS IS INCLINED TO ADDRESS VOLUNTARY CLAIMS FOR FOODS THAT ARE NOT WITHIN THE DEFINITION OF A BE FOOD, AMS SHOULD NOT ENDORSE ON-PACKAGE CLAIMS THAT INGREDIENTS ARE DERIVED FROM OR SOURCED FROM BE CROPS.

We support voluntary labeling and believe that AMS has correctly provided a mechanism to allow regulated entities to voluntarily disclose information concerning BE Foods that are exempted from mandatory disclosure (e.g., small food manufacturers). We also respect regulated entities' right to make other claims regarding BE Foods consistent with federal law. However, we do not support any voluntary labeling scheme linked to a BE crop list that would allow regulated entities to use on-package text or a symbol to indicate that a non-BE Food was derived from or sourced from a BE crop.

First, creating such a voluntary program exceeds AMS's statutory authority. The NBFDS grants the USDA Secretary authority to establish a mandatory BE disclosure standard and to establish requirements and procedures necessary to carry out the standard.[51] In enacting the NBFDS, Congress made clear that "the definition of bioengineering is set in statute and establishes the scope of the disclosure standard."[52] Thus, if a food is excluded from or does not meet the definition of a BE Food it is not within the scope of the NBFDS and within the USDA Secretary's authority to further regulate. Second, allowing such on-package text would effectively rewrite the statutory definition of a BE Food to a food that is derived from or sourced from a BE crop, a definition Congress expressly rejected. Both the market and the consumer will assume that the derived from or sourced from text means the food is BE, which is both false and misleading. This contradicts Congress's purpose that there be a uniform standard for disclosure. There is simply not enough room on a label to fully explain that while certain ingredients may have been derived from a BE crop, the food itself is not a BE Food. Finally, even if AMS were inclined to allow non-BE Foods to have on-package derived from or sourced from text, it is not a logical outgrowth of this rulemaking and therefore would require a separate notice and comment proposal to comply with the Administrative Procedures Act.

We are not opposed to regulated entities providing additional information about the source of their ingredients, provided that the information is placed in context and is not misleading. We believe such information can be provided through the QR code/Smart Label, website, etc. which many food manufacturers are already providing. We see little need for AMS to regulate in this area.

IV. AMS SHOULD ADOPT A 5 PERCENT THRESHOLD THAT ALLOWS THE INTENTIONAL USE OF SMALL QUANTITIES OF BE FOODS (ALTERNATIVE 1-C).

AMS requests comment on three proposed thresholds, two of which would allow the inadvertent or technically unavoidable presence of genetic material at either a 0.9 percent or 5 percent level in food (Alternatives 1-A and 1-B). The third threshold would allow regulated entities to use BE ingredients up to 5 percent of the total weight of the product (Alternative 1-C). While the threshold AMS adopts does not directly impact refined ingredients because they contain no

[51] NBFDS §293(a).
[52] Senate Report at 3.

modified genetic material, it does impact how the technology is viewed by consumers and global trading partners. Thus, given its impact on the current and future use of the technology, we recommend AMS to adopt Alternative 1-C because it supports biotechnology, appropriately balances disclosure, market dynamics, and international trade, and is consistent with other U.S. regulatory programs, including the USDA Organic Program which allows up to 5 percent of non-organically produced agricultural ingredients.

There is no scientific basis for any threshold because biotechnology does not raise health, safety or nutrition concerns.[53] Accordingly, thresholds are simply a tool to create a differentiation in the market place to provide a marketing advantage to non-BE products. Thresholds are arbitrarily established mainly to drive consumers away from the technology and create non-tariff trade barriers to imported biotech commodities to protect domestic producers who do not have access to the technology.[54] As a world leader, and a leader in biotechnology, AMS must provide sound rational for its threshold and not acquiesce to standards set by other countries that attempt to oppose or stigmatize the technology. It is also important to keep in mind that "Congress intend[ed] for the NBFDS to be technology neutral."[55] Other countries are closely watching what the U.S. will do in these regulations and it will likely influence their internal discussions regarding acceptance and disclosure.

Of the thresholds that have been established world-wide, a 5 percent threshold is the most supportive of bioengineering[56]. It is the lowest cost, lowest liability approach that results in consumer savings. It also has the least impact on the domestic and international value chain and is less of a burden on our developing foreign suppliers. It is the most compatible with our North American trading partners, Mexico and Canada, neither of which require disclosure. Finally, it is the closest to technology neutral of the mandatory categories.

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[53] *See e.g.*, USDA Foreign Agricultural Service, European Union 28, Agricultural Biotechnology Annual, December 6, 2016 at 20, 37 (noting that "the EC continues to pursue inconsistent and unpredictable approaches regulating the technology. Due to the strong emotional and ideological stance taken by EU consumers and nongovernmental organizations (NGOs) on biotechnology, born in many ways out of the misleading information provided by anti-biotechnology groups, legislation adopted by the EC as well as the process surrounding the approval for cultivation and use of GE crop varieties has suffered," and further noting that "different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage . . . and communication campaigns to heighten public fears."), available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf.

[54] The European Union's moratorium on approving new genetically modified food illustrates the point. In 2003, the U.S., Canada, and Argentina challenged the moratorium as unfair protectionist measures prohibited by the General Agreement on Tariffs and Trade (GATT). The Panel concluded that "the European Communities applied a general de facto moratorium on approvals of biotech products between June 1999 and 29 August 2003." *See* European Communities – Measures Affecting the Approval and Marketing of Biotech Products. WTO Document WT/DS291R (29 September 2006).

[55] Senate Report at 4.

[56] Japan, South Africa, Indonesia, Vietnam, and Thailand have all adopted a 5% threshold.

Importantly, a 5 percent threshold is consistent with other U.S. regulatory programs. The USDA Organic Program allows up to 5 percent of non-organically produced agricultural ingredients which are not commercially available in organic form.[57] If an organic consumer product can retain the organic label with up to 5 percent non-organic content, then the NBFDS should be set at 5 percent as well. Indeed, federal courts have held that consumers hold products labeled organic to a higher standard than even products labeled natural.[58] Having the same 5 percent threshold reduces consumer confusion and avoids any implication that biotechnology is less safe or less desirable and therefore must be treated more stringently than organic products. In addition, the grain trade has coalesced around a 5 percent low-level presence threshold, although there isn't an international standard.

To be clear and to avoid any misunderstanding, USDA says "[t]he use of genetic engineering, or genetically modified organisms (GMOs), is prohibited in organic products."[59] However, "[t]here aren't specific tolerance levels in the USDA organic regulations for GMOs. As such, National Organic Program policy states that trace amounts of GMOs don't automatically mean the farm is in violation of the USDA organic regulations. In these cases, the certifying agent will investigate how the inadvertent presence occurred and recommend how it can be better prevented in the future."[60]

In contrast, Alternatives 1-A and 1-B that allow only the inadvertent or unavoidable presence of genetic material treat BE ingredients as contaminants. For more than 20 years the U.S. has battled foreign countries that inhibit or reject U.S. exports because of their overly restrictive biotechnology standards, based principally on fear (the precautionary principle), not science.[61] This has resulted in higher food costs to foreign consumers and less sustainable food production. In many instances, these restrictive thresholds are used as a non-tariff trade barrier to imports to protect their domestic producers from U.S. competition.

Moreover, the Non-GMO Project, whose stated mission is to "to change the way our food is grown and made," has a 0.9 percent per ingredient threshold above which a product cannot bear its Non-GMO Project verified label.[62] That is not Congress's intent. Congress made clear that the NBFDS cannot "denigrate biotechnology," which is precisely the Non-GMO Project's

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[57] USDA Labeling Organic Products, https://www.ams.usda.gov/sites/default/files/media/Labeling%20Organic%20Products.pdf.

[58] See e.g., Pelayo v. Nestle USA Inc., 989 F. Supp. 2d 973, 979 (C.D. Cal. 2015).

[59] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[60] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[61] See also "In the EU, different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage (destruction of research trials and cultivated fields), and communication campaigns to heighten public fears." Page 37, USDA Foreign Agricultural Service, European Union 28, Agricultural Biotechnology Annual, December 6, 2016. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf.

[62] Non-GMO Project, https://www.nongmoproject.org/about/mission/.

undeniable objective to drive BE foods out of the market.[63] The Non-GMO Project describes GMOs as "contaminates" and "threats to the supply chain."[64] To adopt the same threshold used by the Non-GMO Project is unsupportable and unacceptable to the American farmers and scientific community that embrace biotechnology. AMS should also carefully consider the potential consequences of a 0.9 percent percent "European-style" unintentional presence threshold (Alternative 1-B) could have on American agriculture.[65] In Europe, "consumers rarely find GE labels on food, because many producers have changed the composition of their products to avoid losses in sales. Indeed, although products undergo a safety assessment and labels are simply there to inform consumers, they are often interpreted as warnings, and producers expect labeled products to fail in the market."[66]

In conclusion, AMS will determine whether the United States will continue to treat the presence of bioengineered substance in food as a "non-disparaged low-level presence ingredient" or a "contaminant." Alternative 1-C is the only threshold that will (1) allow the United States to remain a world leader in the production of BE crops, (2) minimize impacts on the value chain, (3) minimize regulatory burden on farmers, and (4) promote sustainability. Any lower threshold would treat BE ingredients as a contaminant and not be technology neutral and would "denigrate biotechnology" in contradiction of Congress.[67]

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[63] See Non-GMO Project's webinar description that discusses one of the proposed threshold alternatives as "[a]llow[ing] an unreasonably high 5% threshold for GMO contamination in ingredients" : https://www.nongmoproject.org/blog/comment-on-the-national-bioengineered-food-disclosure-standard/

[64] See Non-GMO Project's webinar description and webinar that discusses one of the proposed threshold alternatives as "[a]llow[ing] an unreasonably high 5% threshold for GMO contamination in ingredients" : https://www.nongmoproject.org/blog/comment-on-the-national-bioengineered-food-disclosure-standard/

[65] According to the USDA's own FAS GAIN report, "Until the 1990's, the European Union (EU) was a leader in research and development of biotech plants. Under pressure from anti-biotech activists, EU and Member State (MS) authorities have developed a complex policy framework that has slowed down and limited research, development, and commercial production of biotech products."[65]

[66] USDA, Foreign Agricultural Service, Global Agricultural Information Network, EU-28, Agricultural Biotechnology Annual, Report SP1743 (2017) at 36.

[67] Senate Report at 2.

ATTACHMENT

List of Ingredients	Contains or May Contain
Natural Flavor	corn
Citric Acid	corn
Spice	corn
Lecithin (soy)	soy
Riboflavin (Vitamin B2)	yeast
Corn Syrup	corn
Xanthan Gum	corn
Natural and Artificial Flavor	corn
Corn Starch	corn
Mono and Diglycerides of Fatty Acids	corn
Modified Starch (corn)	corn
Vitamin C	corn
Yeast	yeast
Modified Food Starch	corn, soy, potato
Vinegar	corn
Flour	corn
Artificial Flavor	corn
Sodium Citrates	corn
Yeast Extract	yeast
Malt Flour (barley)	corn
Flavor	corn
Molasses	corn
Soybeans	soy
Brown Sugar	Corn
Distilled Vinegar	corn
Sodium Carboxymethylcellulose	corn
MSG	corn
Corn Syrup Solids	corn
Malic Acid	corn
Rice Flour	rice
Vanilla	corn
Corn	corn
Corn Flour	corn
Soy Flour	soy
Potatoes	potato
Potato Starch	potato
Sorbic Acid	corn
Vanilla Extract	corn
Glycerin or Glycerol	corn
Sodium Oleyl Lactylate	corn
Seasoning	corn
Sodium Erythorbate	corn

Polyoxyethylene Sorbitan Monostearate	corn
Tocopherols Concentrate, mixed	corn
Apple	apple
Artificial Vanilla Flavor	corn
Rice	rice
Flour (bleached)	corn
Baking Powder	corn
Corn Meal	corn
Polysorbate 80	corn
Fructose	corn
Hydrolysed Soy Protein	soy
Soy Protein	soy
Erythrosine	corn
Diacetyltaric & Fatty Acid Esters of Glycerol	corn
Soy Sauce	soy
Soy Protein Isolate	soy
Autolysed Yeast Extract	corn, yeast
Apple Juice Concentrate	apple,
Sorbitol	corn
Canola	canola
Cellulose Powder	corn
Hydrolysed Corn Protein	corn
Cider Vinegar	apple
Pineapple	pineapple
Fumaric Acid	corn
Flour (unbleached)	corn
Potassium Citrates	corn
Cottonseed	cotton
Breadcrumbs	corn
White Vinegar	corn
Mustard	corn
Propylene Glycol Propan	corn
Herbs and Spices	corn
Acetic Acid	corn
Propylene Glycol Mono- and Di-Esters or Propylene Glycol Esters of Fatty Acids	corn
Barley Malt	corn
Natural Vanilla Flavor	corn
Sorbitan Monostearate	corn
Rice Starch	rice
Cheese (sour cream)	corn
Alcohol	corn
Torula Yeast	corn, yeast
Rice Syrup	rice
Calcium Lactate	corn
Sodium Lactate	corn

Vitamin E	corn
Rice (brown)	rice
Potato Flour	potato
Crust	corn
Barley Malt Extract	corn
Rice Flour (brown)	rice
Rice Crisps	rice
Vegetables	corn
Gum Base	soy
Alpha-Tocopherol	corn
Malt	corn
Pineapple Juice Concentrate	pineapple

Attachment 2

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March 14, 2008

Comment and summary in reference to reports of analysis

CD59558 through CD59605 from 06/20/06

CD96333 through CD96369, CD69376 through CD96399 from 04/17/07

Customer: Sugar Industry Biotech Council, 1101 15th St. NW Suite 600, Washington DC 20005-5017, Attn: Charles W. Baker, council member responsible for collating and submitting samples.

Project 1

Various samples of sugar were analyzed for the presence of DNA. A polymerase chain reaction (PCR) test specific for DNA from ubiquitous plant cell organelles called plastids served as an indicator for the presence of plant DNA in general. Since this PCR procedure detects plastid DNA irrespective of the plant species, it was an appropriate procedure to examine sugar derived from various sources for the presence of DNA. PCR is generally more sensitive than other commonly used means of specific or unspecific detection of DNA. Therefore a PCR test appeared suitable to attempt the detection of residual trace amounts of DNA.

Forty-four samples described as commercially available sugar from different sources were analyzed:

- Six samples of organic cane sugar, from Europe, South America and the United States
- Seven samples of turbinado / muscovado sugar, from Africa, Mauritius, Mexico and the United States
- Sixteen samples of white beet sugar from Canada, Europe and the United States
- Fifteen samples of white cane sugar from Africa, Australia, Canada, Caribbean, Europe, Japan and the United States

In addition, four reference samples of analytical grade sucrose were tested. All forty-four samples of commercial sugar and the four reference samples tested negative. It was concluded that plant DNA could not be detected in any of these samples of sugar.

Project 2

From a commercial sugar production process using industrially grown and harvested H7-1 Roundup Ready® sugarbeets, samples of eight different initial, intermediate and final products were taken. These samples ranged from sliced, raw sugarbeets to commercial white sugar. Each different type of product was sampled at the beginning, half way through, and towards the end of Roundup Ready® sugarbeet processing (twenty-four samples total). Eurofins GeneScan inspection personnel on site verified the sampling process and chain of custody for samples submitted to the laboratory.

For control purposes, a respective set of twenty-four samples was taken from a production run using conventional sugarbeets. Thirteen of the sugar samples from Project 1 were used as additional control samples.
All samples were analyzed for the presence of DNA sequences indicative of H7-1 Roundup Ready® sugarbeet. The respective real-time PCR method was developed and in-house validated by Eurofins GeneScan, together with KWS

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SAAT AG, Einbeck Germany and Monsanto Company, St. Louis, MO. The method was validated in a collaborative trial conducted by the European Commission Joint Research Centre - Community Reference Laboratory[1].
All samples were also tested for the presence of the particular novel protein CP4-EPSPS, which confers Roundup tolerance to the H7-1 Roundup Ready® sugarbeet plant. A commercially available protein test kit for CP4-EPSPS was used[2].

As expected, neither the 24 samples from processing of conventional sugar beets nor the 13 other sugar samples from Project 1 showed any detectable CP4-EPSPS protein nor H7-1 DNA sequences.

The results for the 24 samples of H7-1 Roundup Ready® sugarbeet and respective products are summarized in the table below. Results for the three samples of the same product taken at different times were identical in each case.

Table: Test results for H7-1 derived samples

Samples (set of 3 each)	Detection of H7-1 DNA	Detection of CP4-EPSPS Protein
Sliced Sugarbeet	**Yes**	**Yes**
Pressed Pulp	Yes	No
Dried Pulp	Yes	No
Raw Sugarbeet Juice	Yes	Yes
Thin Sugarbeet Juice	No	No
Thick Sugarbeet Juice	No	No
White Sugar	**No**	**No**
Molasses	**No**	**No**

The CP4-EPSPS Roundup Ready® protein was detected in all three samples of sliced, raw H7-1 Roundup Ready® sugarbeets and all three samples of raw sugarbeet juice. CP4-EPSPS Roundup Ready® protein could not be detected in the respective sugar, molasses and pulp samples processed from the Roundup Ready® sugarbeets.

H7-1 DNA was detected in sliced, raw H7-1 Roundup Ready® sugarbeets as well as in raw sugarbeet juice and in pulp from H7-1 Roundup Ready® sugarbeets. However, H7-1 could not be detected in the samples of commercial white sugar and molasses made from H7-1 Roundup Ready® sugarbeets.

Eurofins GeneScan, Inc.

Dr. Frank Spiegelhalter
Executive Vice President

[1] Event-specific method for the quantitation of sugar beet line H7-1 using real-time PCR
http://gmo-crl.jrc.it//summaries/H7-1-Protocol%20Validated.pdf
[2] TraitChek Lateral Flow Strip – Sugarbeet Seed Application Guide; Part Number 7000014
Strategic Diagnostics Inc., 111 Pencader Dr, Newark, DE 19702

United States Beet Sugar Industry

July 3, 2018

United States Department of Agriculture
Agricultural Marketing Service
Docket Clerk
1400 Independence Avenue, SW
Room 4543 - South
Washington, DC 20250

Submitted via www.regulations.gov

RE Proposed Rule – National Bioengineered Food Disclosure Standard – Doc. No. AMS-TM-17-0050 (83 Fed. Reg. 19860 (May 4, 2018))

Dear Sir/Madam:

The attached comments are submitted on behalf of the United States Beet Sugar Industry representing all of the 10,000 progressive family farmers of sugarbeets in 11 states, who own all nine farmer cooperatives (22 factories), the cooperatives' employees, seed producers and the scientists that are engaged in the production and processing of sugarbeets. We produce 56% of the sugar grown in the U.S. We raise sugarbeets on 1.2 million acres, provide 100,000 jobs and generate $10.6 billion for the U.S. economy. We proudly provide the highest quality of sugar for both the safety of our food supply and the food security of our nation. The sugarbeet is one of the best suited plants for use in biotechnology and we have produced 100% bioengineered plants since 2015.

We appreciate the opportunity to comment on the USDA Agricultural Marketing Service's ("AMS") proposed rule to implement the National Bioengineered Food Disclosure Standard, Pub. L. 114-216, (the "NBFDS" or "Act"). We applaud AMS for attempting to address stakeholders' competing views on the scope of the NBFDS by setting forth a number of options for the final rule. Our overriding concern, however, is that some of the options being considered, if adopted, have the potential to harm the U.S. Beet Sugar Industry and stifle American farming innovation by presuming that foods like beet sugar contain genetic material[1] when sound science shows they do not. Above all else, AMS must ensure that the NBFDS is a marketing standard, not a health, safety, or nutritional standard. Congress expressly recognized that "the

[1] We support AMS's proposed definition of "bioengineered substance" that incorporates the statutory definition of ''bioengineering,'' which means ''matter that contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature." Throughout this comment we refer to the statutory term "genetic material" to mean "bioengineered substance" as AMS proposes to define that term.

comprehensive federal review process has determined that foods produced using bioengineering are safe and not materially different in any way from those made using other methods."[2]

As members of the Coalition for Safe Affordable Food we support many of the Coalition's comments and recommendations on the NBFDS. However, we are not aligned with the Coalition on several issues of critical importance to the U.S. Beet Sugar Industry, most importantly on the Coalition's position that AMS should not exclude refined ingredients under Position 1 and instead adopt Position 2 with the undetectable DNA factor and condition. Creating any presumption, even unintentionally, that beet sugar produced from transgenic sugarbeets is different and less desirable than its conventional counterparts or cane sugar is not supported by science, is contrary to the intent of the NBFDS, imposes a costly and discriminatory burden on the industry, and has harmful economic impacts throughout the supply chain. It also creates consumer confusion and increases consumer prices for identical products. As we explain in detail herein, for these reasons we urge AMS to exclude highly refined ingredients, and in particular refined sugar, from the scope of the NBFDS.

We also strongly disagree with the Coalition's recommendation to create a voluntary labeling program that would allow on-package labeling for non-BE Food products, such as beet sugar, with text such as "derived from" or "sourced from" a bioengineered crop. As we discuss in section V herein, any such "derived from" text was expressly rejected by Congress and is misleading to the consumer because it fails to fully explain that while a product may be derived from a bioengineered crop the food itself is not bioengineered.

Finally, we disagree with the Coalition's proposal that AMS adopt a dual threshold comprised of a 0.9% threshold for intentional presence, as measured in the finished food product, and a 5% threshold for unintentional presence, as measured by a particular ingredient. Rather, we urge AMS to adopt Alternative 1-C, allowing the intentional use of BE ingredients up to 5% of the weight of the finished product because it supports biotechnology, appropriately balances disclosure, market dynamics, and international trade, and is consistent with other U.S. regulatory programs, including the USDA Organic Program which allows up to 5% of non-organically produced agricultural ingredients.

Respectively submitted,

American Sugarbeet Growers Association

U.S. Beet Sugar Association

Amalgamated Sugar Company

American Crystal Sugar Company

Big Horn Basin Beet Growers Association

Big Horn County Sugar Beet Growers Association

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[2] S. Rep. No 114-403 (2016 ("Senate Report") at 2.

California Beet Growers Association, Ltd.
Colorado Sugarbeet Growers Association
Elwyhee Beet Growers Association
Idaho Sugar Beet Growers Association
Michigan Sugar Company
Minn-Dak Farmers Cooperative
Montana-Dakota Beet Growers Association
Nebco Beet Growers Association
Nebraska Sugar Beet Growers Association
Nyssa-Nampa Sugarbeet Growers Association
Red River Valley Sugarbeet Growers Association
Sidney Sugars, Inc.
Spreckels Sugar Company
Southern Minnesota Sugar Cooperative
Southern Montana Sugarbeet Growers Association
Western Sugar Cooperative
Wyoming Sugar Company, LLC
Beet Sugar Development Foundation
American Society of Sugar Beet Technologists
Sugar Industry Biotech Council

United States Beet Sugar Industry

Comments on National Bioengineered Food Disclosure Standard Proposed Rule

Table of Contents

EXECUTIVE SUMMARY

In enacting the NBFDS, Congress expressly defined a bioengineered food ("BE Food") as one that "contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques; and (B) for which the modification could not otherwise be obtained through conventional breeding or found in nature" and provided guiding principles for its implementation, which include that:

(1) the NBFDS not treat bioengineered food differently than its non-bioengineered counterpart,

(2) AMS "take every effort to minimize the impacts [of the NBFDS] on growers, handlers, processors, manufacturers, distributors, retailers and consumers,"[3]

(3) AMS minimize the impacts on all aspects of the domestic and international value chain,[4] and

(4) AMS provide "exemptions and other determinations under which a food is not considered a bioengineered food."[5]

Adhering to these principles, we discuss in detail below the following points and recommendations:

- **AMS should not include refined ingredients in the definition of a BE Food (Position 1).**[6] Position 1 is supported by numerous scientific studies demonstrating the absence of genetic material from sugar and AMS's own economic analysis showing that excluding refined sugars and oils from the definition of a BE Food would not reduce the number of

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[3] Senate Report at 8.

[4] *Id.*

[5] *Id.*

[6] AMS continues to refer to processed sugars and oils as "highly refined ingredients." However, the more appropriate term is simply "refined ingredients." Highly processed or refined ingredients typically refer to multi-ingredient mixtures processed to the extent that they are no longer recognizable as their original plant/animal source, e.g., candy, tomato sauce, ice cream, etc. In contrast, when a single isolated food component, such as sugar, is obtained by extraction or purification using physical or chemical processes, it is typically referred to as "refined." *See e.g.,* Poti, J.M., *et al.*, Is the degree of food processing and convenience linked with the quality of food purchased by US households?, 101 *Am. J. Clin. Nutr.* 1251-1262 (June 2015). For these reasons, we urge USDA to use the term "refined ingredients" when referring to single food components such as sugar.

foods subject to disclosure and would be far less costly than requiring product testing to prove the absence of genetic material.

- **If AMS is not inclined to exclude refined ingredients as a group from the definition of a BE Food under Position 1, AMS should at a minimum exclude refined sugar from the definition**. AMS has before it seven published peer-reviewed studies demonstrating the lack of genetic material in refined sugar, as well as testing results from each of the 22 U.S. and one Canadian beet sugar processing factories showing there is no transgenic DNA or protein in the refined sugar extracted from transgenic sugarbeets. This body of science is conclusive and is more than sufficient for AMS to exclude refined sugar from the definition of a BE Food under Position 1. Australia, New Zealand, Japan, Malaysia, South Korea, and Brazil have all relied on this well-established body of science to conclude that refined sugar does not contain genetic material and therefore is not subject to their mandatory BE labeling laws.

- **If AMS is inclined to include refined ingredients in the definition of a BE Food under Position 2, AMS must adopt the undetectable DNA factor and condition.** Including highly refined ingredients, and particularly beet sugar, in the definition of a BE Food without providing a mechanism to exclude products that do not contain genetic material is contrary to Congress's express intent that the NBFDS apply only to foods that contain genetic material. It also treats a food like beet sugar differently than its non-bioengineered counterpart when they are molecularly identical. Disparate treatment of identical products is discriminatory, misleading, and has significant economic impacts on consumers, growers and the entire supply chain.

- **AMS's proposed list of BE Foods confuses BE Foods and crops and creates a presumption that foods "derived from" certain crops are BE Foods contrary to Congress's intent that a bioengineered food "contain genetic material."** We understand and support AMS's objective to create an easily referenced list to facilitate compliance with the NBFDS. However, creating lists of highly adopted and not highly adopted BE Foods by reference to bioengineered crops, which is intended to serve as the "linchpin" for determining whether a regulated entity needs to disclose a BE Food, is not only contrary to Congress's intent that a BE Food contain genetic material, it renders Position 1 and the undetectable DNA factor and condition superfluous. Rather, AMS should adopt a BE ingredient list. Exhibit 2 of the RIA, modified to reflect ingredients excluded from the scope of the NBFDS, i.e., refined ingredients, enzymes, is an easy to understand list that would facilitate compliance with the NBFDS without creating false presumptions or contravening the intent of the NBFDS that a BE Food is one that contains genetic material. Alternatively, AMS could use Table 5 from the RIA which lists the top 50 ingredients that would likely trigger disclosure, provided it eliminates from the list those products excluded from the definition of a BE Food, e.g., sugars, oils, enzymes. This is a far better way for regulated entities to make disclosure decisions because most food manufacturers, and especially small food manufacturers, do not know what crops many ingredients are derived from. The RIA itself supports this approach.

- **If AMS maintains its lists of highly adopted and not highly adopted crops, AMS should remove sugarbeet from the list.** The sugarbeet is not a food within the meaning of the NBFDS; it is grown only for the purpose of producing refined sugar, which under any reading of the NBFDS cannot be considered a BE Food for human consumption. The sugarbeet is the only transgenic crop that produces a single food for human consumption that is conclusively shown, in multiple independent studies, to not contain genetic material. Thus, including the sugarbeet on the list of highly adopted BE Foods creates a false and misleading presumption that refined sugar is a BE Food subject to the mandatory disclosure requirements. Creating a false presumption is contrary to the express will of Congress, is discriminatory and misleading, and has harmful effects to consumers, the industry and throughout the supply chain.

- **If AMS is inclined to address voluntary claims for foods that are not within the definition of a BE Food, AMS should not endorse on-package claims that ingredients are "derived from" or "sourced from" BE crops.** We support food manufacturers' desire to be transparent and disclose additional information concerning ingredients that are not BE Foods under the NBFDS. If AMS is inclined to create any safe harbors, which is not the intent of the law or within the scope of the proposed rule, or provide guidance for such claims, endorsing on-package claims that ingredients are "derived from" or "sourced from" BE crops would create confusion as consumers would presume that sourced or derived from means the food is bioengineered. Not only would this be misleading to consumers, it would defeat Congress's objective to achieve national uniformity in the labeling of BE Foods. Rather, if sourced or derived from claims are made, they should be provided through other means, such as an electronic or digital link, that allows complete and truthful information to be provided.

- **AMS should adopt a 5% threshold that allows for the intentional use of small quantities of BE ingredients.** The threshold AMS establishes impacts how biotechnology is viewed by consumers and global trading partners. A 5% threshold supports biotechnology, appropriately balances disclosure, market dynamics, and international trade, and is consistent with other U.S. regulatory programs, including the USDA Organic Program which allows up to 5% (low level presence) of non-organically produced agricultural ingredients.

I. AMS SHOULD ADOPT POSITION 1 AS THE DEFINITION OF A BIOENGINEERED FOOD

The Preamble to the proposed rule discusses two competing views on whether refined foods, such as refined sugar, should be included within the scope of the NBFDS and invites comment on three specific issues: (1) additional studies that address the presence of genetic material in refined foods, (2) the cost of implementation, including whether the scope of foods subject to the NBFDS would lower costs to affected entities, and (3) which position is the better interpretation of the statutory definition. We address each of these issues below to demonstrate that AMS should adopt Position 1 because it is grounded in science, does not impose unnecessary and unreasonable economic burdens on consumers, food manufacturers, supply chain distribution and transportation systems, or the beet sugar industry, does not decrease the number of foods subject to the NBFDS, and is the better interpretation of the statutory definition of a bioengineered food.

A. The Science is Conclusive: Refined Sugar Produced from Transgenic Sugarbeets Does Not Contain Genetic Material and Therefore Should Be Excluded from the Definition of a BE Food

Refined sugar is defined by FDA as sucrose obtained by crystallization from sugarcane or sugarbeet juice that has been extracted by pressing or diffusion, then clarified and evaporated, which is of a purity suitable for its intended use.[7] In the United States, cane and beet sugar is refined to a purity of 99.9%, thus removing all impurities, including genetic material.[8] For this reason alone, creating any presumption that refined sugar is a BE Food is erroneous. Moreover, the numerous studies discussed below confirm that refined sugar does not contain genetic material.

1. *The Peer-Reviewed Scientific Literature Establishes the Lack of Genetic Material in Refined Sugar*

AMS correctly cites to a number of studies that demonstrate the absence of genetic material in refined sugar. These include a study conducted by German scientists that examined the fate of DNA and protein during the standard purification steps of the sugar extraction process from both conventional sugarbeets and sugarbeets genetically engineered with the coat protein CP21 to confer resistance to a certain virus. (Klein, J., *et al.* 1998).[9] This study is particularly important because it not only failed to detect DNA and protein beyond the early raw juice stage of the

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[7] 21 C.F.R. § 184.1584.

[8] The remaining 0.1% is made up of carbohydrates such as glucose, fructose and raffinose, as well as organic and inorganic salts of sodium, potassium, calcium and magnesium.

[9] Klein, J., Altenbuchner, J., and Mattes, R., Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugarbeets. *J. of Biotechnology*, 60: 145-153 (1998).

refining process, it estimated that the beet sugar clarification process had the potential to reduce the amount of sugarbeet DNA by a factor of ten to the fourteen (a hundred trillion or 0.00000000000001), which exceeds the total amount of DNA present in sugarbeets. AMS also cites to Oguchi, *et al.* (2009) that also found that sugarbeet plant DNA is degraded and removed early in the sugar extraction process and is therefore not present in the finished sugar.[10] The Oguchi study was the basis upon which Japan exempted beet sugar from its mandatory GMO labeling requirements.[11]

With respect to sugar produced from sugarcane, AMS correctly cites to Joyce, *et al.* (2013) and Taylor *et al.* (2009) demonstrating the absence of genetic material in refined cane sugar.[12] In addition, Pauli *et al.* (2000), did not find DNA in either raw or refined cane sugar.[13]

The science is further confirmed by a study published in March 2018. (Cheavegatti-Gianotto, *et al.* 2018).[14] Specifically, Brazilian researchers examined whether sugar produced from sugarcane genetically modified to express the Cry1Ab protein to control the sugarcane borer (*Diatraea saccharalis*) contained transgenic material. The study found that clarified juice, molasses, and raw sugar showed no detectable levels of Cry1Ab protein. Similarly, no heterologous DNA was detected in clarified juice and downstream products including raw sugar. As the researchers conclude, the results are in agreement with the results of other studies that

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[10] Oguchi, T., *et al.*, Investigation of residual DNAs in Sugar from Sugar beet (*Beta vulgaruis L.*), *J. Food Hyg. Soc. Japan*, 50: 41-46 (2009), *available at* https://www.jstage.jst.go.jp/article/shokueishi/50/1/50_1_41/_pdf.

[11] In Japan, processed foods that contain detectable amounts of transgenic DNA or proteins must be labeled to indicate that genetically modified ingredients are used. Japan does not require sugar from transgenic sugarbeets s to be labeled because the refined sugar does not contain transgenic DNA or proteins. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf.

[12] Joyce, P.A., Dinh, S-Q., Burns, E.M., and O'Shea, M.G. (2013), "Sugar from genetically modified sugarcane tracking transgenes, transgene products and compositional analysis," Proc. Int. Soc. Sugar Cane Technol." Vol. 28, pp 1-9; Joyce, P.A., Sedl, J.M. and Smith, G.R. (1999), "Laboratory crystallized sugar from genetically engineered sugarcane does not contain transgene DNA", Proc. Aust. Soc. Sugar Cane Technol., Vol. 21, pp. 502.

[13] Pauli *et al* (2000) Extraction and Amplification of DNA from 55 Foodstuffs. Mitt. Lebensm. Hyg. 91: 491-501.

[14] Cheavegatti-Gianotto, A., *et al.* "Lack of Detection of Bt Surgarcane CRY1Ab and Nptll DNA and Proteins in Sugarcane Processing Products Including Raw Sugar (2018), Frontiers in Bioengineering and Biotechnology, Vo. 6, Art. 24 (2018).

investigated the degradation of specific DNA fragments inserted into genetically modified sugarcane (NptII) and glyphosate-resistant sugarbeet (CP4 EPSPS) that reported the complete elimination of the inserted DNA during processing to refined sugar (Klein *et al.*, 1998; Oguchi *et al.*, 2009; Joyce *et al.*, 2013). Brazil, as the largest producer of cane sugar, relied on the Cheavegatti-Gianotto study to determine that sugar produced from genetically modified sugarcane is a "chemically defined pure substance" that does not fall within the scope of Brazil's Biosafety Law and therefore "is not a genetically modified organism or a derivative thereof." The determination is attached as Attachment 1.

Importantly, the Brazilian study refutes any suggestion that the science is inconclusive about whether refined sugar contains genetic material. In the proposed rule, AMS cites Cullis *et al.* (2014)[15] as one study commenters claim shows that minute quantities of sugarcane DNA were detected in raw sugar (not for human consumption) after industrial milling prior to refining.[16] Commenters do not understand the sugar refining process and misinterpret the scientific findings.

Understanding the steps in the sugar refining process is critical to correctly interpreting the studies. The schematic in Figure 1 illustrates the steps in both the beet and cane refining processes and identifies the points in the process where studies have tested for genetic material. While there is some variability among the studies as to precisely when the DNA and protein are no longer detectable, largely based on the DNA extraction methods used, primer selection, and polymerase chain reaction ("PCR") conditions and fragment length, all studies demonstrate that the genetic material is removed early in the refining processes for both beet and cane sugar.

First, with respect to the Cullis study, as the schematic shows, raw sugar is not refined cane sugar. Raw sugar is produced at a sugar mill as the feedstock to the cane refining process. Raw sugar is not sold for human consumption in the United States. As FDA explains, raw sugar is "the intermediate food product as it leaves the sugar factory mill for further refinement in sugar refineries before use as food. In general, raw sugar is unsuitable for human food use because it contains extraneous impurities which are removed in the refining process."[17] At the refinery,

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[15] Cullis, C., Contento, A., Schell, M., DNA and Protein Analysis throughout the Industrial Refining Process of Sugar Cane. International Journal of Agricultural and Food Research, North America, 3, jul. 2014. Available at: https://www.sciencetarget.com/Journal/index.php/IJAFR/article/view/437.

[16] AMS also cites to one study that purports to have found genetic material in all stages of crude soybean oil processing. We defer to the oil processors to address the merits of the study, but we observe that detection was only possible by using primer combination RRS-3J1 and RRS-3J3 to amplify the NOS terminator, which fall outside the coding region for EPSPS (the glyphosate tolerance gene).

[17] FDA's Compliance Policy Guide (CPG), CPG 515.400 (revised March 1995).

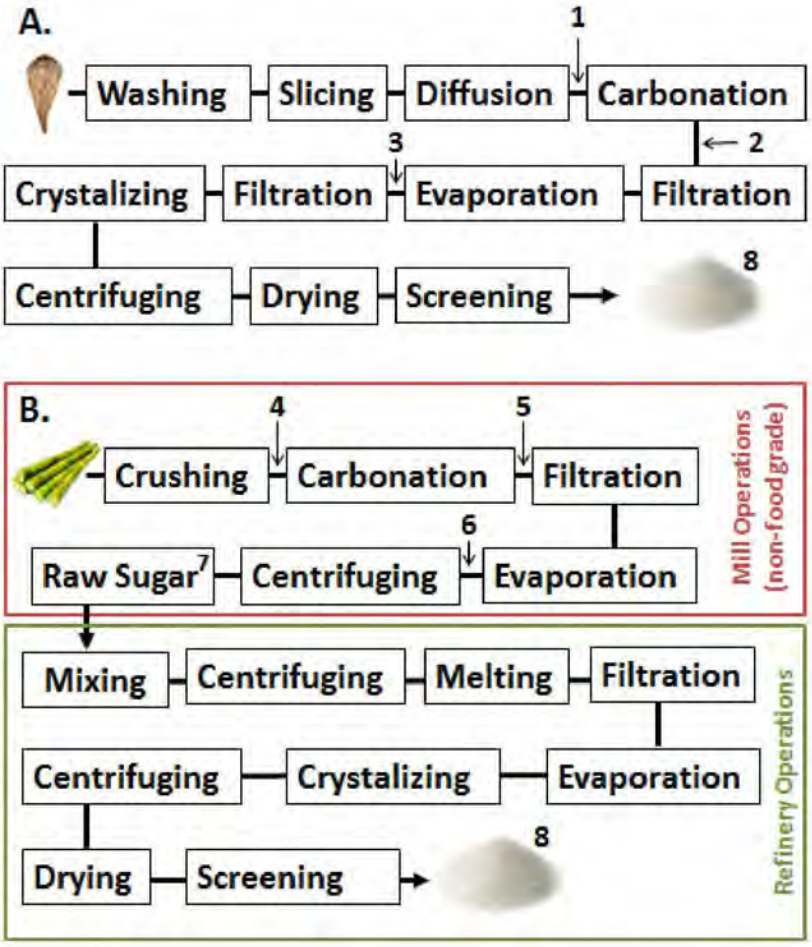
raw sugar undergoes a number of refining steps, including cooking, filtering, evaporation, crystallization, centrifuging, and drying to produce refined cane sugar.

Second, Cullis did not detect fully coding regions of DNA or functional proteins in raw sugar, but rather highly degraded fragments. For example, the PCR amplification of target sequences in raw sugar was only successful using 1 of 4 primer combinations with the shortest amplification length (less than 300 basepairs), suggesting the larger coding region was fragmented and therefore unable to be amplified. Further, regarding protein presence the authors explain "[raw sugar] showed little or no evidence of bands or high molecular mass material suggesting proteins in these fractions are fragmented" as well as the fact that a "…majority of staining material accumulated in a low molecular mass smear…further supports the conclusion that proteins in these later fractions may be significantly degraded."

Finally, and most importantly, as the Cullis study itself demonstrates, even if there is genetic material in the raw sugar, the refining process eliminates it altogether ("PCR failed to detect any sugarcane DNA in refined sugar.").[18] As Cullis concluded, the study's failure to detect DNA in the refined sugar is consistent with previous studies on the detection of DNA through the refining process (Joyce *et al.* (2013), Klein, *et al.* (1998), Oguchi, *et al.* (2009).

[18] Cullis, *et al.* at 14.

Figure 1 Schematic of Beet and Cane Refining Processing Identifying Points Where Samples Have Been Taken to Test for Genetic Material



Beet processing **(A)** occurs all in one food-grade facility. Cane processing **(B)** occurs in two separate facilities: the mill (red box) and the refinery (green box). None of the products produced by the cane mill are considered or handled as food grade products.

Samples at various points in the refining processes have been analyzed to evaluate the presence of absence of DNA/proteins. Those samples for sugarbeet **(A)** include: Raw Juice **(1)**, Thin Juice **(2)**, Thick Juice **(3)** and Refined Sugar **(8)**. Samples for sugarcane **(B)** include: Raw Juice **(4)**, Clarified Juice **(5)**, Syrup **(6)** and Refined Sugar **(8)**. None of the studies have found genetic material in Refined Sugar **(8)**.

2. *Extensive Studies Conducted by the Beet Sugar Industry Establish the Lack of Genetic Material in Refined Beet Sugar*

AMS should also acknowledge three studies conducted by the beet sugar industry that similarly show the absence of DNA and protein in refined beet sugar. While these studies have not been published in the scientific literature, they were conducted using methodologies validated according to Codex Alimentarius guidelines by an ISO/ICE 17025 accredited laboratory. In the first study conducted in 2008 samples were collected from eight different points in the refining process [three samples each at the beginning (sliced beet, pressed pulp, dried pulp), middle (raw, thin, and thick juice, and end (refined sugar and molasses)] at one processing facility. The study demonstrated that while transgenic DNA and the CP4-EPSPS protein[19] was detected in the raw sugarbeet and the raw juice (Point 1 on Figure 1), it was not detected at any other subsequent point in the refining process. Thus, consistent with Klein *et al.* (1998), the study confirmed that the transgenic DNA and CP4-EPSPS protein are removed early in the process at the clarification stage during the transformation from raw juice to thin juice. The study is provided in Attachment 2.

In the second study, multiple samples of sugar produced from transgenic and conventional sugarbeets and sugarcane from around the world were analyzed for the presence of plant (plastid) DNA. More specifically, the study sampled organic sugar from Europe, South America and the U.S.; turbinado/muscovado sugar from Africa, Mauritius, and the U.S.; white beet sugar from Canada, Europe, and the U.S. (including sugar produced from transgenic sugarbeets); and white cane sugar from Africa, Australia, Canada, the Caribbean, Europe, Japan, and the U.S.[20] No plant DNA was detected in any of the samples, thus again confirming the Klein *et al.* (1998) findings that the clarification process effectively removes *all* plant DNA (by a factor of 10^{14}). *See* Attachment 2.

In 2014, the Beet Sugar Development Foundation conducted a third study of all U.S. and Canadian beet sugar factories. Sixty-nine samples of refined sugar were collected from all North American beet sugar factories (three random samples from each of the 22 U.S. factories and the one and only Canadian factory) by the same independent analytic firm to test for any presence of transgenic DNA and the CP4-EPSPS protein. *All 69 samples of commercial sugar tested negative for transgenic sugarbeet DNA, as well as the CP4-EPSPS protein.* The results are provided in Attachment 3. This comprehensive study reaffirmed the 2008 study and is consistent with the scientific literature that shows that there is no transgenic DNA or protein in the sugar extracted from transgenic sugarbeets.

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[19] The CP4-EPSPS protein confers Roundup® tolerance to the H7-1 Roundup Ready® sugarbeet plant.

[20] Forty-four samples of sugar were analyzed, as well as four samples of laboratory pure (analytical grade) sucrose.

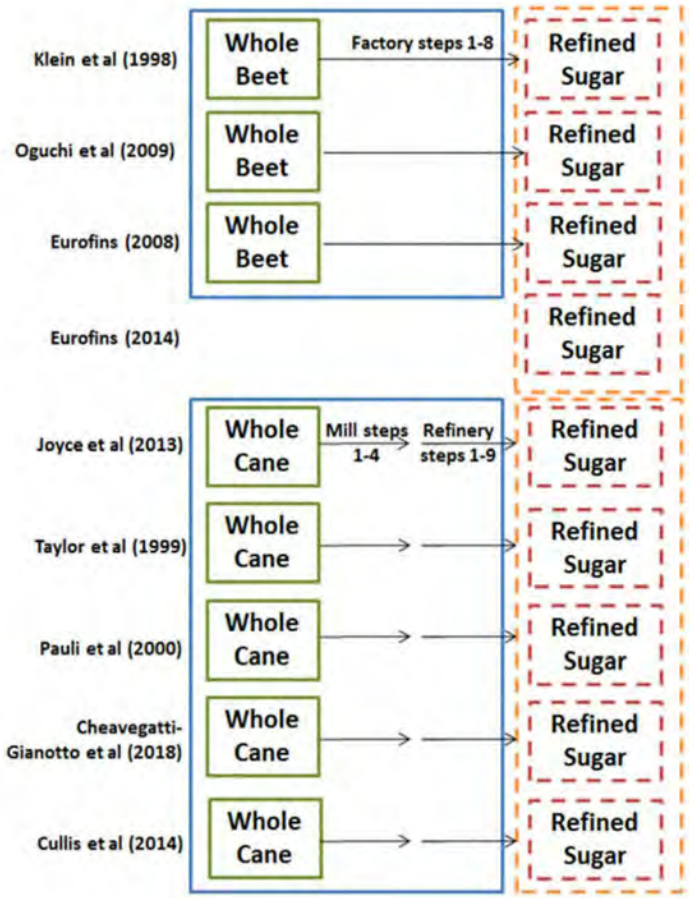
3. *Summary of the Science Supporting Excluding Refined Sugar from the Definition of a Bioengineered Food*

To negate any doubt or misunderstanding of the science, Figure 2 provides a visual summary of the studies examining DNA and protein degradation during the sugar refining process. Because the studies use various terms to refer to similar stages of the refining process, the stages are aligned vertically to provide consistency across studies.

Figure 2 shows that all studies conclude that refined sugar, which is 99.9 percent sucrose, does not contain DNA or protein. These findings are not only scientifically sound, they make logical sense. Any product that is refined to a purity of 99.9% under continuous high heat and in the presence of native nucleases will not contain extraneous impurities or genetic material. Indeed, Klein *et al*. (1998) and other researchers explain that these two factors are responsible for eliminating genetic material from refined ingredients. Even if it is assumed that the remaining 0.1% is genetic material, which it is not, refined sugar would not fall within the definition of a BE Food under AMS's strictest threshold (0.9%).

AMS therefore has before it seven peer-reviewed published studies demonstrating the lack of genetic material in refined sugar, as well as testing results from each of the 22 U.S. and one Canadian beet sugar processing factories showing there is no *transgenic* DNA or protein in the sugar extracted from transgenic sugarbeets. This body of science is more than sufficient for AMS to exclude refined sugar from the definition of a bioengineered food under Position 1. Australia, New Zealand, Japan, Malaysia, South Korea, and Brazil have relied on one or more of these studies to conclude the refined sugar does not contain genetic material and therefore is not subject to their mandatory BE labeling laws.

Figure 2 Visual Summary of Science Demonstrating Lack of Genetic Material in Refined Sugar



4. ***There is No Rational Basis to Include Refined Sugar in the Definition of a BE Food and Exclude Other Food Products and Ingredients that May Contain Genetic Material***

AMS proposes to exclude from the definition of a BE Food incidental additives such as enzymes, which are bioengineered. The Coalition for Safe Affordable Food is also requesting that (1) incidental additives, processing aids, secondary direct additives; (2) food derived from insects or microorganisms that grow or feed on a bioengineered substrate, such as a bioengineered crop or other substance; (3) enzymes; (4) ingredients derived via fermentation regardless of whether the microorganisms used in the fermentation are derived using rDNA technology, and (5) food products with medicinal or supplementary applications be excluded from the definition of a BE Food, and (6) unpackaged BE Foods, e.g., bulk foods and fresh produce. Each of these proposed and requested exclusions are food products and ingredients that are likely, or in the case of bulk foods and fresh produce are certain, to contain genetic material. While we do not object to these food products and ingredients being excluded from the definition of a BE Food under the NBFDS, we are concerned that there is a willingness to exclude certain foods and ingredients that contain some level of genetic material, albeit small, but an unwillingness to exclude refined sugar from the definition of a BE Food when scientific evidence unequivocally demonstrates that refined sugar contains no genetic material at all. Such disparate treatment is not rationally related to the purpose of the NBFDS. Nor is it scientifically or legally justified.

We therefore request and urge that at a minimum AMS adopt Position 1 with respect to refined sugars in the event that AMS is not inclined to exclude refined ingredients as a group from the definition of a BE Food under Position 1.

Creating a presumption that sugar produced from transgenic sugarbeets is different and less desirable than its conventional counterparts is not truthful, is misleading to consumers, contrary to the purpose of the NBFDS, and has harmful economic impacts throughout the supply chain.

We therefore urge that section 66.1 of the regulations expressly exclude refined sugar as follows:[1]

Bioengineered food means—

(1) Subject to the factors, conditions, and limitations in paragraph (2) **and exclusions in paragraph** (3) of this definition, a food that contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and for which the modification could not otherwise be obtained through conventional breeding or found in nature.

(2) A food that meets the following factors and conditions is not a bioengineered food.

(i) An incidental additive present in food at an insignificant level and that does not have any technical or functional effect in the food, as described in 21 CFR 101.100(a)(3) or any successor regulation.

(ii) [Reserved].

(3) Refined sugar produced from bioengineered sugarbeets or sugarcane is not a bioengineered food.

B. Including Refined Ingredients in the Definition of a BE Food Imposes Unnecessary and Unreasonable Economic Burdens on Consumers, Food Manufacturers, Supply Chain Distribution and Transportation Systems, and the U.S. Beet Sugar Industry

In its Regulatory Impact Analysis ("RIA"), AMS analyzed three scenarios for the scope of the NBFDS: (Scope 1) all foods and dietary supplements that have been produced through bioengineering (including refined oils and sugars); (Scope 2) all foods and dietary supplements excluding sugars and oils; and (Scope 3) foods where the genetic material cannot be detected are excluded. As we understand it, Scope 2 equates to Position 1 described in the Preamble, Scope 1 equates to Position 2 without the adoption of the undetectable DNA factor and condition, and Scope 3 equates to Position 2 with the proposed undetectable DNA factor and condition.

The RIA demonstrates that Position 1/Scope 2 (excluding refined ingredients) does not result in fewer food products being subject to the NBFDS, nor does it impose unreasonable costs. However, the RIA's conclusion that the costs of Position 2/Scope 1 are the same as Position 1/Scope 2 does not consider all costs "stretching back to the farm" that would be incurred if refined ingredients like beet sugar were presumed to be a BE Food.

We show below that creating any presumption that beet sugar is a BE Food results in product deselection and price differentials. Our concerns about product deselection and price differentials are validated by a recent survey conducted by the International Food Information Council Foundation (IFIC),[21] which shows that "[a] majority of respondents (53%) say they are less likely to consume food if they know it contains BE ingredients."[22] Furthermore, consumers' willingness to pay for identical products with no-BE disclosure versus products with a BE disclosure decreased prices by up to 15%.[23] When the costs related to product deselection and price differentials are considered, Position 1/Scope 2 (excluding refined ingredients) is the lowest cost option. With respect to Scope 3 (the undetectable DNA factor and condition), the RIA confirms that it results in far fewer products being subject to the NBFDS and imposes far higher testing costs on the industry. For this reason alone, AMS should adopt Position 1 over Position 2 with the undetectable DNA factor and condition.

1. *Excluding Refined Ingredients from the Definition of a Bioengineered Food Does Not Decrease the Number of Products Subject to the NBFDS*

One of the principal arguments raised in opposition to excluding refined ingredients from the definition of a BE Food is that it would significantly decrease the number of foods subject to the

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[21] IFIC Foundation Survey (2018) available at https://www.foodinsight.org/sites/default/files/GMO-foods-survey-results-FINAL.pdf. IFIC surveyed 1002 respondents between May 18-27, 2018 regarding the proposed NBFDS;

[22] *Id.* at 11.

[23] *Id.* at 26 showing the price consumers were willing to pay for a product with no disclosure and an identical product with a BE disclosure was $2.96 and $2.51, respectively.

NBFDS. Some have even suggested there could 80 percent fewer products labelled as a bioengineered food. The RIA squarely refutes these claims.

As the RIA explains, the concept of nesting recognizes that most foods subject to the NBFDS are multi-ingredient foods, any one of which could potentially trigger disclosure under the NBFDS. The RIA therefore evaluated the number of food labels potentially subject to the NBFDS with and without refined sugars and oils included.[24] The RIA found that excluding refined sugars and oils did not result in any noticeable difference in the number of labelled products subject to the NBFDS.[25] The RIA further found that dietary supplements are even less sensitive to the exclusion of refined oils and sugars, finding that only 0.5% of products required to be labeled under Scope 1 would be excluded under Scope 2. In other words, refined sugars and oils are not the ingredients that drive disclosure.

In stark contrast, the RIA demonstrates that adopting the undetectable DNA factor and condition, which would apply to many more foods than just refined foods, results in only 45% of labels being be subject to the NBFDS. Indeed, Exhibit 2 of the RIA demonstrates that only 28 ingredients would be exempt under Position 1/Scope 2, while 98 ingredients would be exempt under Scope 3 (undetectable DNA).

Accordingly, excluding refined sugars and oils under Position 1 has no meaningful effect on the number of food labels subject to the NBFDS and therefore should not be a determining factor in AMS choosing Position 2 over Position 1. Adopting the undetectable DNA factor significantly reduces the number of food labels subject to the NBFDS and, as discussed below, imposes unnecessary costs.

2. *The RIA Does Not Address the Market and Agricultural Impacts that Flow from Presuming Refined Sugar is a BE Food Under Position 2/Scope 1*

The legislative history of the NBFDS makes clear that "the Secretary, when determining the amounts of a bioengineered substance that may be present in food, or the threshold requirement, shall *minimize the impacts on all aspects of the domestic and international value chain,*" as well as "minimize the impacts on growers, handlers, processors, manufacturers, distributors, retailers, and consumers.[26] Moreover, the NBFDS "is not intended to increase the costs of food manufacturing or changes in distribution or handling." Congress's intent that the NBFDS not disrupt domestic and international supply chains is reinforced by E.O. 13777, which established a federal policy to alleviate unnecessary regulatory burdens. Creating any presumption that beet

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[24] RIA at 51.

[25] *Id.* (finding that under Scope 1, 66% of labels would be subject to the NBFDS and under Scope 2, 64% of food labels would potentially be subject to the NBFDS).

[26] Senate Report at 4, 8.

sugar is a BE Food when it does not contain genetic material exacerbates impacts on growers, handlers, processors and the domestic and international value chain.

(a) The RIA fails to consider price impacts of presuming beet sugar is a BE Food under Position 2 when it is identical to all other refined sugar products

The RIA requests comment on the potential market reaction to the NBFDS and in particular, solicits evidence of market reaction to products presumed to be BE Foods. The impact of the Vermont law on beet and cane sugar prices illustrates the harmful impacts that will flow to the beet sugar industry if AMS adopts Position 2.

Historically, the wholesale price per pound of beet sugar and cane sugar has remained steady either with no price differential at all or a one cent or less differential. This is because the market has correctly viewed beet and cane sugar as interchangeable commodities, with prices driven largely by supply and demand. However, as the Vermont law was nearing implementation, the price differential between beet sugar and cane sugar grew substantially because the law required any foods derived from bioengineered crop to be labeled, which caused the market to view beet sugar less favorably.

The Vermont law was scheduled to become effective on July 1, 2016. Figure 3 demonstrates that prior to 2016, the price differential between beet and cane sugar was one cent or less. However, the price differential rose sharply beginning in March 2016 as manufacturers began making supply decisions based on the Vermont law mandating labeling and misleading disclosure text and negatively influencing consumer perceptions that beet sugar was a less desirable product. Indeed, it was well-publicized that one of the biggest sugar users, Hershey's, began reformulating its chocolate products to move from beet to cane sugar.[27] By February 2017, the price differential reached 7.5 cents per pound because of market substitution of cane sugar for beet sugar and concerns over whether cane supplies would be adequate to meet

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[27] Hershey's response to consumer perceptions of GMOs demonstrates that manufacturers would substitute non-BE ingredients for BE ingredients where possible, either by using certified non-GE (including organic) forms of current ingredients, or reformulating products to use alternative ingredients that are not produced in GE forms. As in Europe, food processors and retailers are reluctant to offer for sale food with labels that may (a) frighten or otherwise dissuade some consumers, even though the label is not informative about food safety or the process used to produce it, and (b) provide a target for political action by groups opposed to BE Foods, whose stated intention is to take action if such foods are offered for sale. *See* Alston, Julian and Daniel Sumner, *Proposition 37 – California Food Labeling Initiative: Economic Implications for Farmers and the Food Industry if the Proposed Initiative Were* Adopted, Working Paper, September 3, 2012, http*://www.noprop37.com/wp-content/uploads/2014/09/Alston-Sumner-Prop-37-review.pdf.*

demand.[28] The price differential began decreasing in 2017 in response to the NBFDS and growing market confidence that beet sugar would not be considered a BE Food subject to labeling.

Today, the wholesale price differential between beet and cane sugar has returned to its historical levels of one cent or less per pound, with cane sugar priced at 37 cents per pound and beet sugar at 36 cents per pound.[29] However, if beet sugar is presumed to be a BE Food, even if only for a short period of time, the market reaction will be swift. Any time identical products are differentiated in the market it causes food manufacturers and retailers to restrict their supply chain thereby reducing competition and driving up costs which are eventually passed onto consumers through higher prices. Already the Non-GMO Project label on some cane sugar brands and cane sugar-containing products is being used to suggest and mislead uninformed consumers that cane sugar and products containing it are different and more desirable than beet sugar.

Accordingly, we urge AMS to adopt Position 1 and, at a minimum, exclude refined sugar from the definition of a BE Food to avoid market discrimination that results in higher consumer prices and harmful impacts to the beet sugar industry.

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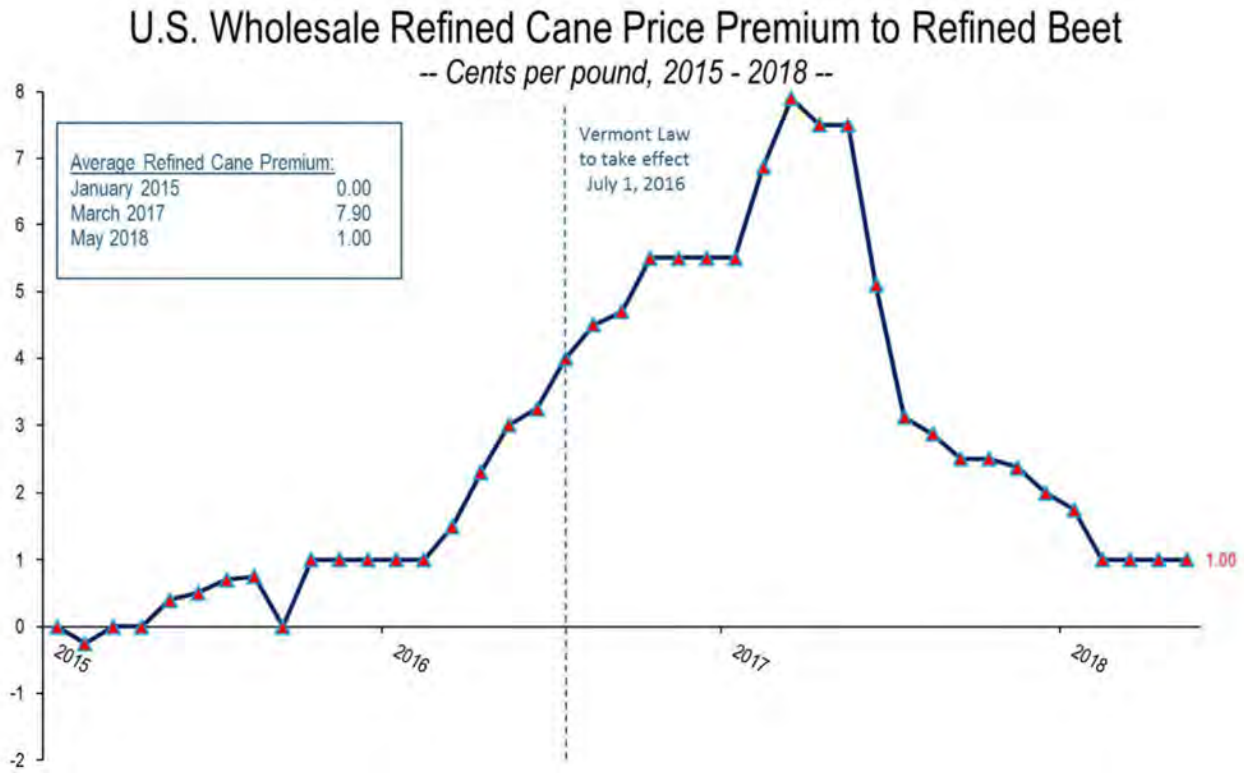
[28] *See also,* USDA, Economic Research Service, "Sugar and Sweeteners Outlook" (May 2016) at 5 (noting a "4.7 percent increase in cane sugar deliveries . . . [and a] 6.9 percent decrease in beet sugar deliveries").

[29] USDA, Economic Research Service, "Sugar and Sweeteners Outlook" (April 2018) at 8.

Figure 3 Price Differential Between Beet and Cane Sugar Due to the Vermont Law



Source: USDA. *Milling and Baking News*. Simple average of lower end of range of quotations for each month. Quotations are weekly. Wholesale refined beet sugar, Midwest markets; wholesale refined cane sugar, Northeast markets. Monthly average prices.

31-X2

(b) Presuming refined sugar is a be food results in supply chain distributions

The RIA addresses a number of impacts associated with manufacturer costs of replacing BE Food ingredients with non-BE Food ingredients to avoid labeling. While the RIA addresses segregation costs, it does not take into account many variables that drive up costs. For example, through a process known in the industry as "swapping," beet and cane sugar is often sold by a particular sugar refiner but delivered to customers from competitors who are geographically closer to the competitor's customers market. This efficient system that reduces transportation costs and congestion on rails and roads, and lowers costs to consumers, would be lost. In addition, private label sugar products for retailers often are supplied by both beet and cane suppliers providing sugar in the same retail package. To avoid different labeling requirements, products would need to be sourced from either the beet or the cane sector which would substantially reduce competition and drive-up costs to consumers. If sourced from both beet and can suppliers, bags of the same product would require different labels, which would also drive up costs. Not only does it disrupt the supply chain, it creates consumer confusion. We therefore believe that the RIA's estimate that segregation costs are 5% above BE market price is a low estimate.

(c) Presuming refined sugar is a be food harms the American farmer

Disruption in the supply chain and disparagement of the technology harms the American sugarbeet farmer because demand for genetically engineered sugarbeets will decline, even though they improve crop yields and are more environmentally sustainable than conventional crops.[30] Indeed, when the Vermont law was enacted many farmers faced uncertainty regarding the future viability of their bioengineered crops which have enabled farmers to adopt production

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[30] "Crop biotechnology has contributed to significantly reducing the release of greenhouse gas emissions from agricultural practices. This results from less fuel use and additional soil carbon storage from reduced tillage with GM crops. In 2012, this was equivalent to removing 27 billion kg of carbon dioxide from the atmosphere or equal to removing 11.9 million cars from the road for one year." GM crops: global socio-economic and environmental impacts 1996-2012. PG Economics Ltd, UK, http://www.pgeconomics.co.uk/page/36/-gm-crop-use-continues-to-benefit-the-environment-and-farmers.

practices that have significantly offset rising costs, including increases in diesel prices,[31] land costs,[32] water costs,[33] industrial energy supplies,[34] seed, fertilizers, and pesticides.[35]

If AMS creates any presumption that beet sugar is a BE Food, the costs stretching back to the farm will be far greater than the RIA estimates. Unlike other crops, there are no non-bioengineered sugarbeets grown for sugar production. Farmers would have to effectively start over to produce a non-bioengineered sugarbeet crop. Not only would all of the cost savings bioengineered sugarbeets provide be lost, the cost to start anew to produce a non-bioengineered sugarbeet would be 2-fold higher than they are today per ton of sugar produced. And, it would take years for farmers to obtain commercially available varieties, cultivars, and registered pesticides necessary to grow a crop. In other words, it would be cost and time prohibitive. This could cause farmers to seek other crop alternatives, which could lead to a major disruption in domestic sugar supplies. Beet sugar processing plants would not be able to run efficiently if there are not adequate supplies of sugarbeets.

Congress instructed AMS to make "every effort . . . to ensure that farmers have access to seed technology and not limit the options available to agricultural production" and directed USDA "to take every effort to minimize the impacts on growers."[36] Adopting Position 2 creates a presumption that beet sugar is a BE Food which is very difficult to overcome in the market even if AMS also adopts the undetectable DNA factor and condition. As we discuss below, AMS's proposed list of BE Foods exasperates the presumption and harms the industry. The risks to the American farmer are far too great for AMS to ignore science and blindly adopt Position 2.

Moreover, impacting the American farmer is directly contrary to E.O. 13790, which established an interagency Task Force to "identify legislative, regulatory, and policy changes to promote in

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[31] US Energy Information Administration, "US Retail Diesel Prices," available at https://www.eia.gov/dnav/pet/hist/LeafHandler.ashx?n=PET&s=EMD_EPD2D_PTE_NUS_DPG&f=M.

[32] The price of land has increased from a national average of $1,830/acre to $3080/acre. *See* USDA, National Agricultural Statistical Service, "Land Values, 2017 Summary," (Aug. 2017), available at https://www.usda.gov/nass/PUBS/TODAYRPT/land0817.pdf.

[33] OECD, "Agricultural Water Pricing: United States," (2010) available at https://www.oecd.org/unitedstates/45016437.pdf.

[34] US Energy Information Administration, "Electric Power Monthly," (March 2018) available at https://www.eia.gov/electricity/monthly/epm_table_grapher.php?t=epmt_5_3.

[35] Univ. of Illinois, "Growth Rates of Fertilizer, Pesticide, and Seed Costs over Time," (July 2016) available at http://farmdocdaily.illinois.edu/2016/07/growth-rates-of-fertilizer-pesticide-seed-costs.html.

[36] Senate Report at 7.

rural America agriculture, economic development, job growth, infrastructure improvements, technological innovation, energy security, and quality of life."[37] In its first report, the Task Force expressly identified technological innovation as one key indicator of rural prosperity. Specifically, with respect to biotechnology, the Task Force noted:

> Biotechnology is another area of U.S. leadership, being a sector that has driven innovation in fuels, chemicals, manufacturing, and agriculture. In 2016, biotech crops were grown on over 170 million acres in the United States, including over 92% of corn, soybean and cotton total acreage, according to the Department of Agriculture's National Agricultural Statistics Service. Globally, the biotechnology sector is a driver of the 'fourth industrial revolution,' and presents and an incredible opportunity for American farmers and rural communities to thrive at the forefront of innovation.[38]

Any mandate that refined foods that do not contain genetic material be subject to the NBFDS undermines the advancement of technology for agricultural production in direct contravention of E.O. 13790. It also perpetuates the misinformation that activists have used for decades to distort the truth about biotechnology, instilling fear in the general public when the global scientific community has repeatedly attested to its safety.[39] Indeed, in making clear that the NBFDS is a marketing standard, not a health, safety, or nutritional standard, Congress expressly recognized that "the comprehensive federal regulatory review process has determined that foods produced using bioengineering are safe and not materially different in any way from those made using other methods."[40] If there were any safety concerns, FDA, not USDA, would act under its authority.

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[37] See Executive Order 13790, "Promoting Agriculture and Rural Prosperity in America" https://www.federalregister.gov/documents/2017/04/28/2017-08818/promoting-agriculture-and-rural-prosperity-in-america.

[38] Report to the President of the United States from the Task Force on Agriculture and Rural Prosperity (Oct. 2017), available at https://www.usda.gov/sites/default/files/documents/rural-prosperity-report.pdf.

[39] *See e.g.*, National Academy of Sciences, The Royal Society of Medicine, WHO, OECD, the American Medical Association, Food and Agriculture Organization of the United States, American Diabetes Association, and the Society of Toxicology.

[40] Senate Report at 4.

(d) Including refined sugar in the definition of a be food impacts foreign beet and cane producers that adopt bioengineered technology to improve environmental impact and sustainability and disrupts international trade.

The United States is the third largest sugar importer in the world, providing access to 41 countries to supply approximately 30% of our sugar market. Any effort to differentiate between beet and cane sugar would cause foreign beet and cane producers to avoid technology that would be better for the environment and increase their efficiency and productivity. This undermines global sustainability objectives.

The United States already imports sugar derived from BE sugarbeets (Alberta) and bioengineered sugarbeets from Ontario, Canada for processing in Michigan. Brazil's government recently approved the world's first commercial bioengineered sugarcane modified to express Bt (Bacillus thuringiensis), which confers resistance to an insect referred to as the cane borer. In March 2018, Brazil also determined that the sugar produced from the bioengineered sugarcane is a "chemically defined pure substance" that does not fall within the scope of Brazil's Biosafety Law and therefore "is not a genetically modified organism or a derivative thereof." *See* Attachment 1. Brazil is by far the largest sugarcane producer and exporter in the world and is the third largest supplier of raw sugar to the United States. Current expectations are that sugar derived from the new variety will reach commercial export markets in 2020. As the world leader in sugarcane production, other cane producing countries look to Brazil for technical advances. For example, Australia and Indonesia are currently developing BE sugarcane varieties with drought resistance, herbicide tolerance, plant development, increased sugar content, and yield.[41] These advances will provide many environmental benefits and increase long term sustainability, which food manufacturers are demanding to ensure sustainability throughout their supply chains. Misguided labeling schemes for refined ingredients, such as sugar, would inhibit such advances and should not be adopted by AMS.

If refined sugar is not excluded from the definition of a BE Food, international trade with Canada would be impacted. Brazil is the largest raw sugar supplier to Canada. (7-year Olympic average is 78% of all raw imports). Canadian companies manufacture sugar-containing products for export to the United States. If refined sugar is considered a BE Food, raw sugar imported from Brazil would have to be segregated from other raw sugars derived from non-bioengineered cane in the Canadian refineries. Also, Canada annually exports around 550,000 short tons of sugar in sugar-containing products to the United States duty free. If refined sugar is considered a BE Food, it would place unnecessary burdens on our trading partners and discourage the adoption of bioengineered crops that are more productive and environmentally sustainable.

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[41] https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Canberra_Australia_8-7-2015.pdf USDA Gain Report on Australia; https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Jakarta_Indonesia_7-14-2015.pdf USDA Gain Report on Indonesia

For these reasons we urge AMS to adopt Position 1 with respect to refined sugars in the event that AMS is not inclined to exclude refined ingredients as a group from the definition of a BE Food under Position 1.

C. Position 1 Implements the Plain Language of the Statutory Definition of a Bioengineered Food

Agency interpretations of statutes they implement are generally considered under the two-part inquiry articulated in *Chevron U.S.A., Inc. v. NRDC*, 467 U.S. 837 (1984). First, if Congress has "directly spoken" to the question at issue," the unambiguous intent of Congress controls. *Pharm. Research & Mfrs. of Am. v. Thompson*, 251 F.3d 219, 224 (D.C. Cir. 2001). If the statute is "'silent or ambiguous with respect to the specific issue," the agency's interpretation is given deference if it is reasonable. *Citizens Coal Council v. Norton*, 330 F.3d 478, 481 (D.C. Cir. 2003) (*quoting Chevron,* 467 U.S. at 843). Here, Congress unambiguously defined a bioengineered food as a "food that contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques." 7 U.S.C.§1639(1)(A). Congress thoughtfully, deliberately and intentionally did not extend the scope of the Act to include ingredients derived from bioengineered crops that do not contain transgenetic material.

The legislative history reinforces the plain language of the statute and makes clear that the definition of a bioengineered food set forth in the statute establishes the scope of the disclosure standard:

> "The Secretary of Agriculture is directed to establish a mandatory uniform national disclosure standard for human food that is or may be bioengineered. For this purpose, *the definition of bioengineering is set in statute and establishes the scope of the disclosure standard.* Congress intends an item of food to be subject to the definition if it contains genetic material that has been modified through in vitro recombinant deoxyribonucleic acid (DNA) techniques and this same modification could not be otherwise obtained through conventional plant breeding or found in nature."[42]

Accordingly, refined foods that do not contain genetic material ***do not*** meet the statutory definition of a bioengineered food. As demonstrated by the science discussed in section I-A, refined sugar indisputably does not contain genetic material and therefore cannot be a bioengineered food within the scope of the NBFDS.

Some groups may argue that Congress defined "bioengineering" in § 291(1) of the Act and gave the Secretary discretion in § 293(a) to define a bioengineered food. They say this reading of the Act is consistent with floor statements made by Members during debate and with a memo from

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[42] Senate Report at 3.

USDA's General Counsel, which some incorrectly describe as a legal opinion. These groups are reading Member statements and the memo out of context. Nevertheless, they cannot supplant the plain language of the NBFDS.

There is no provision in the NBFDS where Congress gave the Secretary the discretion to rewrite the definition of a BE Food from a food that itself contains genetic material to any food derived from bioengineering, a definition Congress expressly rejected. Position 2 modifies the statutory definition of a BE Food by creating a presumption that refined ingredients like sugar are BE Foods because they are derived from BE crops. As discussed further below, AMS's proposed lists of highly adopted and not highly adopted foods amplifies the presumption and further contravenes the statutory definition of a BE Food. The presumption also renders superfluous Congress's direction that the Secretary "determine the *amounts* of a bioengineered substance" that may be present in food to be considered a BE Food because it creates a zero threshold. As the Supreme Court has repeatedly made clear the "plain language" of a statute is the "'primary guide'" to Congress' preferred policy." *Sandoz, Inc. v. Amgen, Inc.*, 137 S. Ct. 1664, 1678 (2017) (quoting *McFarland v. Scott*, 512 U.S. 849, 865 (1994). Here, the plain language makes clear that "bioengineering . . . *with respect to a food, refers to a food* . . . that *contains genetic material*." § 291(1).

Even if the definition of a BE Food were considered ambiguous, which it is not, adopting Position 2 would be an unreasonable interpretation of the NBFDS for four reasons. First, it signals to the market that sugar produced from bioengineered sugarbeets is somehow different or less desirable than sugar produced from sugarcane contrary to Congress's direction that the NBFDS not treat bioengineered food differently from its non-bioengineered counterpart. As discussed in section I -B above, this leads to price differentials and harmful market impacts. *See Motor Vehicle Mfrs. Ass'n of U.S. v. State Farm Mut. Auto. Ins. Co.*, 463 U.S. 29, 43 (1983) (an agency's decision is arbitrary or capricious if it runs counter to the evidence before the agency, relies on factors which Congress did not intend, and/or is not otherwise the product of reasoned decision making.). Second, it creates chaos in the domestic and international supply chain contrary to Congress's direction that AMS minimize the impacts on all aspects of the domestic and international value chain. Third, there is no reasonable rationale for exempting from the definition of a BE Food foods that contain genetic material, such as incidental additives, enzymes, yeasts, and other bioengineered ingredients but include in the definition refined sugar that contains no genetic material whatsoever. Finally, adopting Position 2 and making refined ingredients like sugar subject to the mandatory disclosure requirement compels commercial speech that is not truthful, *see Zauderer v. Office of Disciplinary Counsel*, 471 U.S. 626 (1985) (First Amendment protects commercial speech and protects advertisers from compelled speech), which is also false and misleading under the Food, Drug and Cosmetic Act.

II. IF AMS INCLUDES REFINED INGREDIENTS IN THE DEFINITION OF A BE FOOD UNDER POSITION 2, AMS MUST ADOPT THE UNDETECTABLE DNA FACTOR AND CONDITION.

If, despite the unequivocal evidence that refined sugar does not contain genetic material, AMS is inclined to adopt Position 2, AMS must also adopt the undetectable DNA factor and condition and, as discussed in section IV-C below, make clear at the time the Final Rule is published that

refined sugar is excluded from the definition of a BE Food under the undetectable DNA factor and condition. Including refined beet sugar, in the definition of a bioengineered food without providing a mechanism to exclude it from the definition of a BE Food is contrary to Congress's express intent that the NBFDS apply only to foods that contain genetic material. It also discriminates against refined foods like beet sugar by treating it differently from its non-bioengineered counterpart when the foods are molecularly identical, which leads to the harmful market impacts discussed in section I-B above.

Including refined sugar in the definition of a BE Food, but allowing its exclusion under the undetectable DNA factor and condition is confusing and not necessary when the agency has before it multiple scientific studies demonstrating the absence of any genetic material in refined sugar. It sends misleading messages to consumers by creating a presumption that refined sugar is a BE Food but is excluded from the mandatory disclosure requirements. And, as the IFIC survey shows, it places an onerous burden on the industry to overcome the presumption, to educate consumers on the benefits of bioengineered crops, and to gain consumer acceptance of the technology. Indeed, the U.S. Beet Sugar Industry was a founder of "A Fresh Look" which brings farmers from across the country together to educate consumers about the benefits of GMO farming methods, including how bioengineered crops allow farmers to produce food with less water, land, energy and pesticides.[43] A Fresh Look strives to, among other things, promote food marketing practices that address science-based health and environmental benefits — not spread misinformation to justify inflating prices for some foods, while playing on consumer fears to stigmatize other, equally healthy options. AMS should support such efforts, not create misleading presumptions that undermine them.

Finally, AMS notes that it may consider compatibility of the undetectable DNA factor and condition with U.S. trading partners. However, we believe that Position 1 (excluding refined ingredients from the definition of a BE Food) is more compatible with U.S. trading partners than creating a presumption that a refined food like beet sugar is a BE Food but is excluded from mandatory disclosure under the undetectable DNA factor and condition. We are not aware of any country that requires industry to demonstrate through testing that refined ingredients do not contain genetic material prior to determining that the ingredients are not subject to the county's labeling laws. Rather, countries have relied on published studies to determine that refined ingredients are outside the scope of their mandatory labeling laws. As noted above, Japan relied on Oguchi, *et al*. (2009) to exempt beet sugar from its mandatory GMO labeling requirements[44]

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[43] For more information about A Fresh Look, *see* https://afreshlook.org/.

[44] In Japan, processed foods that contain detectable amounts of transgenic DNA or proteins must be labeled to indicate that genetically modified ingredients are used. Japan does not require sugar from transgenic sugarbeets to be labeled because the refined sugar does not contain transgenic DNA or proteins. USDA FAS "Japan, Agricultural Biotechnology Annual, Japan's regulatory system for GE crops continues to improve", https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Tokyo_Japan_7-13-2015.pdf.

and Brazil relied on Cheavegatti-Gianotto, *et al.* (2018)[45] to determine that bioengineered sugarcane is a "chemically defined pure substance" that does not fall within the scope of Brazil's Biosafety Law and therefore "is not a genetically modified organism or a derivative thereof." *See* Attachment 1. We urge AMS to do the same with respect to refined sugar. There is simply no justification for creating a false presumption that refined sugar is a BE Food but is not subject to mandatory labeling requirements when the agency has before it conclusive scientific evidence that refined sugar is not a BE Food within the meaning of the NBFDS.

III. AMS SHOULD ESTABLISH A DEFINITION OF UNDETECTABLE DNA IF THE UNDETECTABLE DNA FACTOR AND CONDITION IS ADOPTED

AMS proposes that compliance with the undetectable DNA factor and condition be demonstrated by records showing that genetic material was not detected through testing performed by a laboratory accredited under ISO/ICE 17025:2017 standards, using methodology validated according to Codex Alimentarius guidelines. We support AMS's framework for standardized laboratory accreditation and rigorous analytical method validation for determining the presence of genetic material.

However, the proposed rule's undetectable standard may be construed as establishing "absolute zero" as the standard for disclosure of refined ingredients, which would impose substantial regulatory burden due to significant substantiation difficulty based on the inherent nature of test methods having established "analytical/detectable zero" criteria, not absolute zero. As previously stated, we urge AMS to adopt Position 1 and exclude refined ingredients from the definition of a BE Food based on well-established science, but if the undetectable DNA factor and condition is adopted, we support the Coalition's recommendation that the final rule establish a "de minimis" level of recombinant DNA (rDNA) at or below which ingredients qualify as refined ingredients not subject to mandatory disclosure. The "de minimis" level should be set at the generally recognized level of detection of 0.1% rDNA. Specifically, the final rule specify that ingredients qualify as refined ingredients not subject to mandatory disclosure if rDNA is at or below a "de minimis" level set at 0.1%, as measured by the relative proportion of rDNA compared to total DNA using a standard DNA control." Where 0.1% is below the level of rDNA detection for some ingredients, we recommend that such ingredients be excluded from the definition of a BE Food based on "the limit of detectability of modified rDNA as determined by results developed and practiced in accordance with the ISO/ICE 17025:2017 standard, using methodology validated according to Codex Alimentarius guidelines."

By establishing a "de minimis" level for refined ingredients, the rule would avoid the substantial regulatory burden associated with an on-going search to substantiate zero genetic material in various ingredients and the regulatory uncertainty that may accompany advances in scientific methods. Recognizing that no accurate method for testing of DNA exists when the overall

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[45] Cheavegatti-Gianotto, A., *et al.* "Lack of Detection of Bt Surgarcane CRY1Ab and Nptll DNA and Proteins in Sugarcane Processing Products Including Raw Sugar (2018), Frontiers in Bioengineering and Biotechnology, Vo. 6, Art. 24 (2018).

content is below 0.1%, many European countries have adopted a "technical zero" of 0.1% rDNA. This burden-reducing strategy is particularly appropriate in this rulemaking where the statute expressly requires a threshold for disclosure and where disclosure is not a matter of public health and safety.

Should AMS believe a "de minimis" level not to be appropriate, AMS should allow and define undetectable rDNA to mean "the level below the limit of detectability of modified rDNA as determined by results developed and practiced in accordance with the ISO/ICE 17025:2017 standard, using methodology validated according to Codex Alimentarius guidelines." The final rule should clearly state that test methodology in accordance with the ISO/ICE 17025:2017 standard and accreditation (specific to rDNA/PCR testing) is required to validate or challenge presence of modified rDNA in an ingredient whether or not the level of its presence is at a uniform "de minimis" level or the limit of detection determined by each tested ingredient. We submit that failure to require use of an appropriate validated and accredited methodology to detect modified genetic material in an ingredient would add significantly to AMS's burden in administering the rule and could undermine the scientific integrity of rule administration.

We also encourage AMS to provide expectations regarding PCR (polymerase chain reaction) testing, as PCR has become the standard analytical tool used for the detection, identification, and quantification of specific DNA sequences, including rDNA. Specifically, we request that AMS establish minimal standards for selecting appropriate PCR primers for each and any rDNA event that would be subject to the definition of bioengineering.

IV. AMS'S PROPOSED LIST OF BE FOODS CONFUSES BE FOODS AND CROPS AND CREATES A PRESUMPTION THAT FOODS "DERIVED FROM" CERTAIN CROPS ARE BE FOODS CONTRARY TO CONGRESS'S INTENT THAT A BE FOOD "CONTAIN GENETIC MATERIAL"

AMS proposes to create two lists of "BE Foods," one for "highly adopted" BE Foods and the other for "not highly adopted" foods. AMS intends that these lists "would serve as the linchpin in determining whether a regulated entity would need to disclose a BE Food under the NBFDS." However, the "BE Food lists" are lists of bioengineered crops - not BE Foods. By creating a list of BE crops, which includes sugarbeet, to serve as the "linchpin" for determining whether disclosure is required makes superfluous any exclusion AMS provides for refined ingredients under Position 1 or under the undetectable DNA factor and condition. Regulated entities will rely on the crop list, not the exclusions under the law to make disclosure decisions. Thus by default, AMS is defining a BE Food as one derived from a bioengineered crop in direct contravention of the NBFDS.

A. AMS Should Create an Ingredient List to Facilitate Compliance with the NBFDS

We understand and support AMS's intent to facilitate compliance with the NBFDS. However, we believe the better way is to create a BE ingredient list, which the RIA has already created through an extensive analysis of food product labels. Exhibit 2 of the RIA, modified to reflect ingredients excluded from the scope of the NBFDS, *i.e.*, refined ingredients, enzymes, is an easy to understand list that would facilitate compliance with the NBFDS without creating false

presumptions or contravening the intent of the NBFDS that a BE Food is one that contains genetic material. Alternatively, AMS could use Table 5 from the RIA which lists the top 50 ingredients that would likely trigger disclosure, provided it eliminates from the list those products excluded from the definition of a BE Food, e.g., sugars, oils, enzymes. This is a far better way for regulated entities to make disclosure decisions because most food manufacturers, and especially small food manufacturers, do not know what crops many ingredients are derived from. The RIA itself supports this approach:

> If the USDA provided a definitive list of final ingredients by type of disclosure (may contain, does contain), manufacturers' analysis would consist of matching their list of ingredients to the list of required disclosures. That would move most, if not all, products into the low cost category. Therefore, all else held equal, the more clarity USDA provides on which ingredients should apply each label type, the higher the potential savings.[46]

To demonstrate that such a list is workable, we provide in Attachment 4 a BE ingredient list based on RIA Exhibit 2, that does not include refined ingredients or enzymes.

B. If AMS Maintains Its BE Food Lists by Reference to Highly Adopted and Not Highly Adopted Crops, AMS Should Remove Sugarbeet from the List.

If AMS insists on creating a list of BE Foods by reference to bioengineered crops, the sugarbeet should not be included on the list for two important reasons. First, the sugarbeet is the only crop that produces a single food for human consumption – refined beet sugar. As shown throughout this comment, refined beet sugar is pure sucrose, which does not contain any genetic material from the sugarbeet. Including the sugarbeet on the list creates the false and misleading presumption that refined beet sugar is a BE Food.

Second, the sugarbeet itself is not a food for human consumption. As part of its review of the transgenic sugarbeet, FDA described the food and feed uses of the sugarbeet and made clear that the sugarbeet is not a food for human consumption.[47] FDA also exempts sugarbeets from its produce rules under the Food Safety Modernization Act because they are not intended for human consumption.[48] Moreover, the Monsanto Technology/Stewardship Agreement, which grants growers a license to use the transgenic sugarbeet seed expressly prohibits growers from planting the sugarbeet seed for any use other than for processing for sugar, for energy production, or for animal feed. For these reasons, AMS's expressed intent that "only foods or products on either of those lists or made from foods on either of the lists would be subject to disclosure under the NBFDS" is arbitrary and not workable.

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[46] RIA at 29.

[47] FDA Biotech Consultation for H1-7 (#90).

[48] 21 C.F.R. § 112.2(a)(1).

C. If AMS Creates a BE Food List that Includes Bioengineered Crops, AMS Must Also Create an Excluded Ingredient List When the Final Rule Is Published

Although we do not believe it is the best approach to facilitating compliance with the NBFDS, if AMS adheres to its proposal that the BE Food list reference bioengineered crops, we support the Coalition for Safe Affordable Food's recommendation that AMS also create an Excluded Ingredients List that identifies those ingredients that are excluded from the scope on the NBFDS either under Position 1 or the undetectable DNA factor and condition. Providing an Excluded Ingredients List is the only way AMS can mitigate the false and misleading presumptions created by a crop list alone. However, because AMS has before it ample evidence that refined sugar does not meet the statutory definition of a BE Food, it is imperative that an initial Excluded Ingredients List be published with the Final Rule and that initial list include refined sugar. If there is any delay between the publication of the Final Rule and the creation of an Excluded Ingredient List, AMS will create confusion in the market and impose an onerous burden on the beet sugar industry to overcome the false and misleading presumption that refined beet sugar is a BE Food. Market and consumer reaction to the Final Rule will be swift and will likely overtake any efforts by the beet sugar industry to correct the erroneous presumption that refined beet sugar is a BE Food. For these reasons, we urge AMS to create a BE Food list of ingredients, not crops.

V. IF AMS IS INCLINED TO ADDRESS VOLUNTARY CLAIMS FOR FOODS THAT ARE NOT WITHIN THE DEFINITION OF A BE FOOD, AMS SHOULD NOT ENDORSE ON-PACKAGE CLAIMS THAT INGREDIENTS ARE "DERIVED FROM" OR "SOURCED FROM" BE CROPS.

We support voluntary labeling and believe that AMS has correctly provided a mechanism to allow regulated entities to voluntarily disclose information concerning BE Foods that are exempted from mandatory disclosure, e.g., very small food manufacturers. We also respect regulated entities' right to make other claims regarding BE Foods consistent with federal law. However, we do not support any voluntary labeling scheme linked to a BE crop list that would allow regulated entities to use on-package text or a symbol to indicate that a non-BE Food was "derived from" or "sourced from" a bioengineered crop.

First, creating such a voluntary program exceeds AMS's statutory authority. The NBFDS grants the Secretary authority to establish a mandatory bioengineered disclosure standard and to establish requirements and procedures necessary to carry out the standard.[49] In enacting the NBFDS, Congress made very clear that "the definition of bioengineering is set in statute and establishes the scope of the disclosure standard."[50] Thus, if a food is excluded from the definition of a bioengineered food it is not within the scope of the NBFDS and within the Secretary's authority to further regulate. Second, allowing such on-package text would

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[49] NBFDS §293(a).

[50] Senate Report at 3.

effectively rewrite the statutory definition of a BE Food to a food that is "derived from" or "sourced from" a bioengineered crop, a definition Congress expressly rejected. Both the market and the consumer will assume that the derived from or sourced from text means the food is bioengineered, which is both false and misleading. Indeed, the IFIC survey validates that "[a] majority of respondents (53%) say they are less likely to consume food if they know [or assume] it contains BE ingredients."[51] Furthermore, consumers' willingness to pay for identical products with no-BE disclosure versus products with a BE disclosure decreased prices by up to 15%.[52] Thus, many consumers would avoid products with a "derived from" or "sourced from" label because they would erroneously assume that those products contain BE ingredients. As we have shown throughout this comment, such a false and misleading presumption is extremely harmful to the beet sugar industry.

This undeniably frustrates Congress's purpose that there be a uniform standard for disclosure. There is simply not enough room on a label to fully explain that while certain ingredients may have been derived from a bioengineered crop, the food itself is not a BE Food. Finally, even if AMS were inclined to allow non-BE Foods to have on-package derived from or sourced from text, it is not a logical outgrowth of this rulemaking and therefore would require a separate notice and comment proposal to comply with the Administrative Procedures Act.

We are not opposed to regulated entities providing additional information about the source of their ingredients, provided that the information is placed in context and is not misleading. We believe such information can be provided through the QR code/Smart Label, website, etc. which many food manufacturers are already providing. We see little need for AMS to regulate in this area.

VI. AMS SHOULD ADOPT A 5% THRESHOLD THAT ALLOWS THE INTENTIONAL USE OF SMALL QUANTITIES OF BE FOODS (ALTERNATIVE 1-C)

AMS requests comment on three proposed thresholds, two of which would allow the inadvertent or technically unavoidable presence of genetic material at either a 0.9% or 5% level in food (Alternatives 1-A and 1-B). The third threshold would allow regulated entities to use BE ingredients up to 5% of the total weight of the product (Alternative 1-C). While the threshold AMS adopts does not directly impact refined sugar, because beet sugar contains no genetic material at all, it does impact how the technology is viewed by consumers and global trading partners. Thus, given its impact on the current and future use of the technology, we urge AMS to adopt Alternative 1-C because it supports biotechnology, appropriately balances disclosure, market dynamics, and international trade, and is consistent with other U.S. regulatory programs,

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[51] IFIC at 11.

[52] IFIC at 26 showing the price consumers were willing to pay for a product with no disclosure and an identical product with a BE disclosure was $2.96 and $2.51, respectively.

including the USDA Organic Program which allows up to 5% of non-organically produced agricultural ingredients.

There is no scientific basis for any threshold because biotechnology does not raise health, safety or nutrition concerns.[53] Accordingly, thresholds are simply a tool to create a differentiation in the market place to provide a marketing advantage to non-bioengineered products. Thresholds are arbitrarily established mainly to drive consumers away from the technology and create non-tariff trade barriers to imported biotech commodities to protect domestic producers who do not have access to the technology.[54] As a world leader, and a leader in biotechnology, AMS must provide sound rational for its threshold and not acquiesce to standards set by other countries that attempt to oppose or stigmatize the technology. It is also important to keep in mind that "Congress intend[ed] for the NBFDS to be technology neutral."[55] Other countries are closely

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[53] *See e.g.*, USDA Foreign Agricultural Service, European Union 28, Agricultural Biotechnology Annual, December 6, 2016 at 20, 37 (noting that "the EC continues to pursue inconsistent and unpredictable approaches regulating the technology. Due to the strong emotional and ideological stance taken by EU consumers and nongovernmental organizations (NGOs) on biotechnology, born in many ways out of the misleading information provided by anti-biotechnology groups, legislation adopted by the EC as well as the process surrounding the approval for cultivation and use of GE crop varieties has suffered," and further noting that "different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage . . . and communication campaigns to heighten public fears."), available at https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf.

[54] The European Union's moratorium on approving new genetically modified food illustrates the point. In 2003, the U.S., Canada, and Argentina challenged the moratorium as unfair protectionist measures prohibited by the General Agreement on Tariffs and Trade (GATT). The Panel concluded that "the European Communities applied a general de facto moratorium on approvals of biotech products between June 1999 and 29 August 2003." *See* European Communities – Measures Affecting the Approval and Marketing of Biotech Products. WTO Document WT/DS291R (29 September 2006).

[55] Senate Report at 4.

watching what the U.S. will do in these regulations and it will likely influence their internal discussions regarding acceptance and disclosure.

Of the thresholds that have been established world-wide, a 5% threshold is the most supportive of bioengineering[56]. It is the lowest cost, lowest liability approach that results in consumer savings. It also has the least impact on the domestic and international value chain and is less of a burden on our developing foreign suppliers. It is the most compatible with our North American trading partners, Mexico and Canada, neither of which require disclosure. Finally, it is the closest to technology neutral of the mandatory categories.

Importantly, a 5% threshold is consistent with other U.S. regulatory programs. The USDA Organic Program allows up to 5% of non-organically produced agricultural ingredients which are not commercially available in organic form.[57] If an organic consumer product can retain the organic label with up to 5% non-organic content, the NBFDS should be set at 5% as well. Indeed, federal courts have held that consumers hold products labeled organic to a higher standard than even products labeled natural. *See e.g., Pelayo v. Nestle USA Inc.*, 989 F. Supp. 2d 973, 979 (C.D. Cal. 2015). Having the same 5% threshold reduces consumer confusion and avoids any implication that biotechnology is less safe or less desirable and therefore must be treated more stringently than organic products. In addition, the grain trade has coalesced around a 5% low-level presence threshold, although there isn't an international standard.

To be clear and to avoid any misunderstanding, "[t]he use of genetic engineering, or genetically modified organisms (GMOs), is prohibited in organic products."[58] However, "[t]here aren't specific tolerance levels in the USDA organic regulations for GMOs. As such, National Organic Program policy states that trace amounts of GMOs don't automatically mean the farm is in violation of the USDA organic regulations. In these cases, the certifying agent will investigate how the inadvertent presence occurred and recommend how it can be better prevented in the future."[59]

In contrast, Alternatives 1-A and 1-B that allow only the inadvertent or unavoidable presence of genetic material treat bioengineered ingredients as contaminants. For over 20 years the U.S. has battled foreign countries that inhibit or reject U.S. exports because of their overly restrictive biotechnology standards, based principally on fear (the precautionary principle), not science.[60]

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[56] Japan, South Africa, Indonesia, Vietnam, and Thailand have all adopted a 5% threshold.

[57] USDA Labeling Organic Products, https://www.ams.usda.gov/sites/default/files/media/Labeling%20Organic%20Products.pdf.

[58] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[59] https://www.ams.usda.gov/publications/content/can-gmos-be-used-organic-products

[60] See also "In the EU, different types of civil society organizations have militated against agricultural biotechnology since it was first introduced in the 1990s. They are generally opposed to economic growth and globalization. They see more risks than opportunities in technical

This has resulted in higher food costs to foreign consumers and less sustainable food production. In many instances, these restrictive thresholds are used as a non-tariff trade barrier to imports to protect their domestic producers from U.S. competition.

Moreover, the Non-GMO Project, whose stated mission is to "to change the way our food is grown and made," has a 0.9% per ingredient threshold above which a product cannot bear its Non-GMO Project verified label.[61] That is not Congress's intent. Congress made clear that the NBFDS cannot "denigrate biotechnology," which is precisely the Non-GMO Project's undeniable objective in order to drive bioengineered foods out of the market.[62] The Non-GMO Project describes GMOs as "contaminates" and "threats to the supply chain."[63] To adopt the same threshold used by the Non-GMO Project is unsupportable and unacceptable to the American farmers that embrace biotechnology. AMS should also carefully consider the potential consequences of a 0.9% percent "European-style" unintentional presence threshold (Alternative 1-B) could have on American agriculture.[64] In Europe, "consumers rarely find GE labels on

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progress and campaign for a broad application of the precautionary principle. Some of them defend an ideal science that would focus solely on understanding phenomena, and not on developing useful and profitable applications; others reject or strongly criticize science and progress, in line with philosophers such as Hans Jonas and Bruno Latour. They are skeptical of new technologies, in general, and for biotechnology specifically they feel it is dangerous, of little public benefit, and developed by companies that seek private profit at the expense of the common good. As part of their political strategy, their actions include lobbying public authorities, acts of sabotage (destruction of research trials and cultivated fields), and communication campaigns to heighten public fears." Page 37, USDA Foreign Agricultural Service, European Union 28, Agricultural Biotechnology Annual, December 6, 2016. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Agricultural%20Biotechnology%20Annual_Paris_EU-28_12-6-2016.pdf.

[61] Non-GMO Project, https://www.nongmoproject.org/about/mission/.

[62] See Non-GMO Project's webinar description that discusses one of the proposed threshold alternatives as "[a]llow[ing] an unreasonably high 5% threshold for GMO contamination in ingredients" : https://www.nongmoproject.org/blog/comment-on-the-national-bioengineered-food-disclosure-standard/

[63] See Non-GMO Project's webinar description and webinar that discusses one of the proposed threshold alternatives as "[a]llow[ing] an unreasonably high 5% threshold for GMO contamination in ingredients" : https://www.nongmoproject.org/blog/comment-on-the-national-bioengineered-food-disclosure-standard/

[64] According to the USDA's own FAS GAIN report, "Until the 1990s, the European Union (EU) was a leader in research and development of biotech plants. Under pressure from anti-biotech activists, EU and Member State (MS) authorities have developed a complex policy framework

food, because many producers have changed the composition of their products to avoid losses in sales. Indeed, although products undergo a safety assessment and labels are simply there to inform consumers, they are often interpreted as warnings, and producers expect labeled products to fail in the market."[65] As shown above in section I-B, the Vermont law, which adopted the 0.9% threshold, caused food companies to reformulate products to avoid disclosure leading in significant price differentials between identical sugar products and impacts on the American farmer.

In sum, AMS will determine whether the United States will continue to treat the presence of bioengineered substance in food as a "non-disparaged low-level presence ingredient" or a "contaminant." Alternative 1-C is the only threshold that will (1) allow the United States to remain a world leader in the production of bioengineered crops, (2) minimize impacts on the value chain, (3) minimize regulatory burden on farmers, and (4) promote sustainability. Any lower threshold would treat bioengineered ingredients as a contaminant and not be technology neutral and would "denigrate biotechnology" in contradiction of Congress.[66]

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that has slowed down and limited research, development, and commercial production of biotech products."[64]

[65] USDA, Foreign Agricultural Service, Global Agricultural Information Network, EU-28, Agricultural Biotechnology Annual, Report SP1743 (2017) at 36.

[66] Senate Report at 2.

Attachment 3

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Comments and Summary in Reference to Reports of Analysis

Customer:

Beet Sugar Development Foundation
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Lab Numbers:

CG11256	CG11257	CG11258	CG11259	CG11260	CG11261
CG11262	CG11263	CG11264	CG11265	CG11266	CG11267
CG11268	CG11269	CG11270	CG11271	CG11272	CG11273
CG11274	CG11275	CG11276	CG11277	CG11278	CG11279
CG11280	CG11281	CG11282	CG11283	CG11284	CG11285
CG11348	CG11349	CG11350	CG11351	CG11352	CG11353
CG11354	CG11355	CG11356	CG11357	CG11358	CG11359
CG11360	CG11361	CG11362	CG11363	CG11364	CG11365
CG11366	CG11367	CG11368	CG11369	CG11370	CG11371
CG11372	CG11373	CG11374	CG11375	CG11376	CG11377
CG11378	CG11379	CG11380	CG11381	CG11382	CG11383
CG11384	CG11385	CG11386			

Sampling Plan:

Sampling was performed by an independent third-party sampling company between July 21st 2014 and August 1, 2012, and sent via courier to Eurofins GeneScan for analysis. Three subsamples of ~100 grams each were pulled directly from a packaging line at each location, at approximately thirty minute intervals. In facilities that were not actively packaging at the time of sample collection, subs were pulled from three different storage locations to increase heterogeneity amongst the subsamples.

Samples of commercially available sugar from each of the following locations were analyzed:

Brawley, CA	Ft. Morgan, CO	Nampa, ID	Paul, ID	Twin Falls, ID
Bay City, MI	Caro, MI	Croswell, MI	Sebewaing, MI	Crookston, MN
East Grand Forks, MN	Renville, MN	Billings, MT	Sidney, MT	Worland, WY
Drayton, ND	Hillsboro, ND	Moorhead, ND	Wahpeton, ND	Scottsbluff, NE
Lovell, WY	Torrington, WY	Taber, Alberta Canada		

Project 1

Sixty-nine (69) samples of beet sugar obtained from twenty-three (23) processing locations across North America were analyzed for the presence of transgenic sugarbeet DNA. A polymerase chain reaction (PCR) test suitable for the detection of trace amounts of DNA was used for this analysis. Realtime PCR specific for DNA from the glyphosate tolerant H7-1 Roundup Ready® sugarbeet served as an indicator for the presence of transgenic DNA in the samples. PCR was performed in parallel with appropriate positive and negative control reactions.

All sixty-nine samples of commercial sugar tested negative for event H7-1 sugarbeet DNA.

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Project 2

The same sixty-nine (69) samples of beet sugar obtained from twenty-three (23) processing locations across North America were analyzed for the presence of the particular novel protein, CP4-EPSPS, which confers Roundup tolerance to the H7-1 Roundup Ready® sugarbeet plant. A commercially available protein test kit for CP4-EPSPS (Romer, Union, MO #7000014) was used for this analysis.

None of the sixty-nine samples showed any detectable CP4-EPSPS protein.

Eurofins GeneScan

E. Pearce Smith
Lab Manager

eurofins | GeneScan

REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/24/14
Report Date: 08/09/14

‡Description: 2000 lb tote east line 07/23/14 9:30
Submitted by: BRAWLEY CA
Lab Number: CG11277
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

Testing Cert. 1940.01

Eurofins GeneScan

Dr. Frank Spiegelhalter
Executive Vice President

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/24/14
Report Date: 08/09/14

‡Description: 2000 lb tote east line 07/23/14 9:30
Submitted by: BRAWLEY CA
Lab Number: CG11278
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

ACCREDITED

Testing Cert. 1940.01

Eurofins GeneScan

Dr. Frank Spiegelhalter
Executive Vice President

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/24/14
Report Date: 08/09/14

‡Description: 2000 lb tote east line 07/23/14 9:30
Submitted by: BRAWLEY CA
Lab Number: CG11279
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

Testing Cert. 1940.01

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Dr. Frank Spiegelhalter
Executive Vice President

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Packaging line 3 Silo 1 sample 1 07/18/2014
Submitted by: BAY CITY MI
Lab Number: CG11378
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025
Testing Cert. 1940.01

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Dr. Frank Spiegelhalter
Executive Vice President

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Packaging line 3 Silo 1 sample 2 07/18/2014
Submitted by: BAY CITY MI
Lab Number: CG11379
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

ISO/IEC 17025

ilac-MRA ACCREDITED

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Packaging line 3 Silo 1 sample 3 07/18/2014
Submitted by: BAY CITY MI
Lab Number: CG11380
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/30/14
Report Date: 08/09/14

‡Description: Bulk rail loading 07/30/14 7:42
Submitted by: BILLINGS MT
Lab Number: CG11363
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/30/14
Report Date: 08/09/14

‡Description: Bulk rail loading 07/30/14 8:12
Submitted by: BILLINGS MT
Lab Number: CG11364
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/30/14
Report Date: 08/09/14

‡Description: Bulk rail loading 07/30/14 8:34
Submitted by: BILLINGS MT
Lab Number: CG11365
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Lower feed table Weibull silo sample 1 07/18/14
Submitted by: CARO MI
Lab Number: CG11381
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Lower feed table Weibull silo sample 2 07/18/14
Submitted by: CARO MI
Lab Number: CG11382
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Lower feed table Weibull silo sample 3 07/18/14
Submitted by: CARO MI
Lab Number: CG11383
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: 50# bags 07/18/14 8:30
Submitted by: CROOKSTON MN
Lab Number: CG11259
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: 50# bags 07/18/14 9:00
Submitted by: CROOKSTON MN
Lab Number: CG11260
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: 50# bags 07/18/14 9:30
Submitted by: CROOKSTON MN
Lab Number: CG11261
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡**Description:** 2000 lb tote filling line 07/28/14 10:20
Submitted by: CROSWELL MI
Lab Number: CG11280
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡Description: 2000 lb tote filling line 07/28/14 10:50
Submitted by: CROSWELL MI
Lab Number: CG11281
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡Description: 2000 lb tote filling line 07/28/14 11:20
Submitted by: CROSWELL MI
Lab Number: CG11282
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Rail Auger 07/18/14 2:00
Submitted by: DRAYTON ND
Lab Number: CG11262
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Rail Auger 07/18/14 2:30
Submitted by: DRAYTON ND
Lab Number: CG11263
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: Rail Auger 07/18/14 3:00
Submitted by: DRAYTON ND
Lab Number: CG11264
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: East Bulk Rail 07/18/14 10:30
Submitted by: EAST GRAND FORKS MN
Lab Number: CG11256
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: East Bulk Rail 07/18/14 11:00
Submitted by: EAST GRAND FORKS MN
Lab Number: CG11257
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/21/14
Report Date: 08/09/14

‡Description: East Bulk Rail 07/18/14 11:30
Submitted by: EAST GRAND FORKS MN
Lab Number: CG11258
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/22/14
Report Date: 08/09/14

‡Description: 50 lb bagging line 07/21/14 1:30
Submitted by: FT. MORGAN CO
Lab Number: CG11268
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

‡The results shown in this report relate solely to the item submitted for analysis.

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/22/14
Report Date: 08/09/14

‡Description: 50 lb bagging line 07/21/14 2:00
Submitted by: FT. MORGAN CO
Lab Number: CG11269
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/22/14
Report Date: 08/09/14

‡Description: 50 lb bagging line 07/21/14 2:30
Submitted by: FT. MORGAN CO
Lab Number: CG11270
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡Description: Rail conveyor belt 07/28/14 8:05
Submitted by: HILLSBORO ND
Lab Number: CG11283
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡Description: Rail conveyor belt 07/28/14 8:35
Submitted by: HILLSBORO ND
Lab Number: CG11284
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 07/29/14
Report Date: 08/09/14

‡Description: Rail conveyor belt 07/28/14 9:10
Submitted by: HILLSBORO ND
Lab Number: CG11285
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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REPORT OF ANALYSIS

Customer: **Beet Sugar Development Foundation**
800 Grant St. Suite 300
Denver, CO 80203
Attn: Thomas K. Schwartz

Date Received: 08/01/14
Report Date: 08/09/14

‡Description: Warehouse bulk tote A 07/31/14
Submitted by: LOVELL WY
Lab Number: CG11372
Commodity: granulated beet sugar

Analysis	Result	Units	Analyzed
PCR Qualitative - H7-1 sugar beet	Negative	NA	08/09/14
Roundup Ready sugar beet LF Immunoassay	Negative	NA	08/09/14

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Review of USDA's Regulatory Impact Analysis of the Proposed National Bioengineered Food Disclosure Standard

For

The Corn Refiners Association

Submitted by:

Policy Navigation Group

August 2018

Section 3 Review and Recommended Improvements for USDA's Regulatory Analysis

In this section, we discuss some of the major issues with the RIA that cause it to underestimate the social costs of the federal action and of the other state mandatory labeling programs. Although the rulemaking covers both food products and dietary supplements, we confine our analysis to the RIA's evaluation of food product costs. We also discuss in this section some of the most significant improvements we believe USDA can and should undertake to inform policy officials and the public. There are also many important issues with some of USDA's data sources such as the FDA's label cost model and rDNA testing costs. We urge USDA to evaluate public comments submitted to USDA on this rulemaking and to FDA on its rulemakings and to address these comments in its final analysis.

Although we find USDA substantially underestimated the burden of its proposal, these omissions also apply to USDA's analysis of the potential baseline scenarios. Therefore, while the overall burden of this action should increase once USDA incorporate the costs below, USDA's action would still remain a net burden reduction regulatory action. However, the increased burden of USDA's rulemaking discussed in this section should encourage USDA to select alternatives -- like the Coalition's -- to reduce the NBFDS regulatory compliance costs.

Consumer Behavior and Ingredient/Production Costs

The RIA examines the social costs of the rulemaking from the perspective of entities that must comply and could face enforcement action if they do not. This perspective, while reasonable, omits some social costs that are borne by consumers directly and then by both consumers and producers as they readjust to a new, post-regulation market equilibrium. By considering the food producer, the RIA's cost methodology departs from typical supply chain cost structures and from how non-genetically modified organism (GMO) food product verification is occurring in today's markets.

We recommend USDA adopt traditional microeconomic analysis of markets. Resting on the established principle of consumer sovereignty, we start with how consumer behavior will change. As discussed in more detail below, consumers directly bear costs to read and to interpret the new label and the disclosure information. Consumers are also confronted in short order with higher prices as firms attempt to recover some of their increased regulatory compliance costs.

In response to the information and higher prices, consumers will adjust their purchases. The RIA reasonably assumes that some consumers will mistakenly interpret the new label as conveying a health warning and will shift to products without a BE label.[23] Supply of non-BE products will then arise to meet this shifting consumer demand. Other shifts are reasonable and are predicted by the laws of supply and demand. For example, consumers will reduce overall purchases of regulated products because of the higher prices; the potential health consequences of this action are discussed below. Consumers will also shift their purchases to other unregulated substitutes like prepared meals. The RIA should follow each of these market changes to capture completely the social costs that flow from the regulation.

Retailers, restaurants, and food producers will respond to this shift in demand by increasing production of non-BE substitutes. As the RIA predicts, suppliers can create new products that are formulated so as to avoid labeling to recapture some of this lost revenue. The RIA estimates a substantial number of new products will be created, 95,000, or over 12 percent of current UPC codes in FDA's 2014 labeling database.[24]

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[23] RIA, pg. 42.

[24] RIA, pg. 47.

To create these new products, the food producer's first attempt will be to purchase non-BE ingredients from their existing supply chain. Ingredient producers will then have the incentive to demonstrate they can sell a verified, non-BE ingredient to meet this increased demand. They are also the market participant that can provide this verification at the lowest cost since they have the greatest knowledge of the ingredient's sources. Based on classic market analysis, we would expect the market participant with the greatest incentive and the lowest cost to generate non-BE ingredients -- and their verification -- for food producers.

This supply dynamic is already occurring; several organizations offer verification services, documentation, and use of a non-GMO brand to ingredient makers and food producers.[25] Today, ingredient manufacturers can pay for third-party certifications that food producers can use to support labeling claims. We believe the regulation will amplify this existing supply chain dynamic rather than disrupt it.

Moreover, this market dynamic is found in many complex supply chains where the manufacturer relies on many different vendors. In recent years, retailers and consumer-facing manufacturers have seen much greater consumer interest in whether products are produced with child labor, from minerals mined in area under conflict, with or containing certain chemicals, from "sustainable" forestry, and many other attributes of how the product was produced. Even without consumer interest in certain product attributes, manufacturers are constantly evaluating vendors' product quality. Manufacturers/retailers have over time established supply chain audit procedures and vendor standards that are supervised through reporting, testing, and factory visits.[26]

Manufacturers will audit and conduct random verification testing even if ingredient suppliers certify that ingredient does not contain BE material. Manufacturers bear the enforcement risk under USDA's proposal. There have been sufficient enforcement cases in other situations where companies have been held liable when vendors provided them fraudulent verification or credits.[27] We would expect manufacturers to conduct some independent testing of their purchased ingredients. However, from how other markets behave, in most cases, manufacturers will find it less expensive to rely on vendor's certification for most ingredient shipments.

The RIA takes a different approach and assigns a great deal of costs to the food manufacturer to evaluate and test all its ingredients. The RIA assumes a one-time administrative cost for every product. This cost includes all of the activity a food producer must expend to ascertain a product's BE status under the regulation. In Scope 1 and Scope 2, the RIA assumes a significant number of these products will require analytical testing. The RIA effectively assumes that some producers will conduct six tests if they produce six products even if the six products use the same ingredient from the same supplier.

This RIA assumption we believe overstates the likely cost of this initial assessment, assuming food producers and their suppliers have enough time before the effective date to establish commercial terms on the level of ingredient verification required. Manufacturers may conduct the initial round of sampling if the compliance date is imminent. Without enough time to source and to verify BE status

[25] See, for example, the services provided by the Technical Advisors for the Non-GMO Project.

[26] See, for example, Accountancy Europe, Assurance for a Sustainable Supply Chain, June 30, 2005, EcoVadis, Global Reporting Initiative, The Sustainability Code, and many other standards and verification procedures.

[27] See discussion at 79 FR 42079.

from suppliers, food producers would likely quickly conduct their own tests.[28] However, once this initial testing is conducted, manufacturers over time will negotiate commercial terms with suppliers to obtain the necessary verification without testing every shipment.

We also note that the RIA observes the rulemaking's total testing costs would be much less expensive if conducted on a per ingredient/per ingredient vendor basis than on a per food item basis in its discussion about Scope 3.[29] We believe this observation arises from mathematic fact, not a result of a particular regulatory option. It will always be less costly to test each individual factor than to test all the combinations of those factors. While multiple ingredient (factor) providers and the possible regulatory thresholds for labeling complicate the mathematics, since firms are already operating in that fashion in the current non-GMO market and in other supply chain attribute markets, it appears more likely that the lowest cost compliance system is for ingredient providers to conduct the necessary testing to provide a concise verification demonstration that many food processors can rely upon for many different foods.

While the RIA's approach may overstate the burden of the initial assessment, assuming there is sufficient time to come into compliance, it ignores the on-going cost ingredient suppliers and food producers will pay to maintain compliance. Manufacturers will require verification information with some frequency, e.g., each shipment, each crop, each year, since they must maintain continuous compliance. The RIA omits these on-going costs.

In addition to testing and verification costs, the RIA does not include the costs for manufacturers to reformulate new products. The RIA makes a strong assumption that non-BE ingredients are always available and are near-perfect substitutes for the original ingredient. There are many reasons why perfect substitutes may not be available or available at a price that consumers are willing to pay. For some infrequently-used ingredients, the market may not be large enough for ingredient suppliers to bear the testing and verification costs to offer a non-BE version. Sudden consumer interest in a food or flavor (e.g., pumpkin spice) may outstrip near-term supply. Further, non-BE ingredients may be available, but not at a price consumer are willing to pay. Food processers may try to reformulate products to lower the retail price by substituting less costly, alternative ingredients.

Finally, the analysis should follow the supply costs downstream to the retail level. Firms cannot introduce 95,000 new products without additional marketing costs. Wholesalers also must store, ship, and manage 95,000 new products. Retailers also cannot fit 95,000 new products on existing shelf space without displacing other products. The RIA states, "It could also be confusing to see two seemingly identical versions of the same product side-by-side on the same grocery shelf. We assume that manufacturers will chose to avoid this confusion and the additional warehouse and slotting costs. We therefore do not include these costs here."[30]

However, this conclusion is not a logical outcome of the RIA's assumptions. The 95,000 new products the RIA assumes must be developed, shipped, and placed on retail shelves. If there is a risk of consumer confusion, producers must pay to advertise and to induce consumers to try their new products. We see no basis to remove these costs as a consequence of the rulemaking.

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[28] The more likely consequence of an abbreviated period between the final rule and the effective date is the cost of multiple label changes. A firm may protective label its product to ensure compliance and then, after testing and supply chain verification is complete, pay for another label change to remove the BE mark.

[29] RIA, pp. 58-59.

[30] RIA, pg. 46.

Recommended Revisions

In general, we recommend that the RIA track market dynamics more closely to estimate social costs. The current approach we believe omits some significant social costs, does not model accurately how costs will be distributed throughout the supply chain, and ignores the on-going costs to maintain compliance. Specifically, we recommend USDA track the market changes from the regulation in the following path from the change in the consumer demand through the changes in the supply chain to meet this consumer demand:

- Consumers spend resources reading, understanding and considering the new information in their purchasing decisions. Based on this expenditure of social resources due to the regulation, consumer demand shifts. One of those shifts are that consumers demand more goods without the BE label.
- Ingredient suppliers to provide non-BE ingredients and appropriate verification to their customers, food manufacturers. The level of appropriate verification will likely vary in the marketplace based on the reputation of the supplier, the regulatory threshold, and the food manufacturer's compliance risk tolerance, level of independent testing, verification, and auditing.
- Food manufacturers institute supply chain audits for non-BE ingredients comparable to those for other attributes like ingredient quality and those established for other purposes (e.g., verifying supplies are not from conflict areas). Food manufacturers introduce new non-BE products by substituting non-BE ingredients and eliminate some conventional products due to falling demand and increased marketing costs. For some products, food manufacturers can only fulfill consumer demand by reformulating products using different non-BE ingredients.
- Food manufacturers and retailers bear additional costs to distribute and to display the additional non-BE products.

There is available data for USDA to revise the analysis and to prepare estimates of the initial verification costs that ingredient suppliers will pay. For example, Table 21 of the RIA gives a range of testing costs for the top 60 ingredients. USDA could extend that approach to all of the relevant ingredients in Exhibit 2. USDA could also identify from commercial data sources and from Census data the number of ingredient suppliers that could possibly offer verified, non-BE ingredients. As mentioned, some vendors today offer non-GMO independent verification and validation services. These per ingredient costs are greater than the testing costs USDA uses in the RIA.[31] While we would expect that these costs to fall once USDA promulgates this regulation, USDA should consider administrative costs for independent verification beyond simply the laboratory testing of the ingredient.[32] The services listed by these third-parties offer a good basis for USDA to construct estimates of verification costs.

Another fundamental difference between our recommended post-regulation market analysis and the approach in the USDA's RIA is on-going costs. While the RIA treats the testing/verification as a one-time cost, the market will require suppliers to bear on-going costs to maintain compliance. Things change; ingredient suppliers and food manufacturers cannot rely on a one-time test and verification for

[31] See, for example, https://www.nongmoproject.org/wp-content/uploads/2016/08/SCS-Cost-Sheet-05-03-2016.pdf

[32] Some of the costs embedded in services today are likely for the license to use the third-party verification's logo on their products. Once USDA establishes its logo for BE foods, these private party labels may lose some of their value. Therefore, after the regulation, these organizations may lower their prices.

all future compliance.[33] We recommend that USDA include on-going verification and some testing costs by ingredient suppliers so that they can demonstrate continual non-BE status for those ingredients.

As a representative calculation, we use USDA's approach in its Scope 3 estimate. We assume that there are 325 ingredients from the ones listed in Exhibit 2A that would require on-going verification of their BE status under the regulation. We assume that 40 ingredient suppliers supply all 325 ingredients and conduct their own testing on each ingredient once per month. We assume these ingredient suppliers pay $100 per ingredient to third parties for verification, comparable to current prices charged today. We use the per ingredient testing cost range of $153-$431 given in the RIA. Table 4 shows the costs of the regulation's on-going cost to ingredient suppliers to demonstrate compliance to food producers.

Further, as happens today, manufacturers audit their supply chain routinely to ensure regulatory compliance and to ensure brand integrity. Different management techniques and international standards offer different triggers for audits - temporal ones (e.g., every year), process ones (e.g., every time there is a significant process change), and others. We recommend USDA select a well-recognized approach from the process management or insurance field and assume food manufacturers adopt it in response to the regulation. For this representative calculation, we assume manufacturers' costs are ten percent of ingredient suppliers' costs.

Table 4. On-Going Testing and Verification Costs

Number of possible BE ingredients	325
Number of ingredient suppliers	40
Number of tests per ingredient per year	12
Cost of third-party verification per ingredient	$100
Lower Bound Testing Cost	$153
Upper Bound Testing Cost	$431
Lower Bound Annual Ingredient Verification Costs	$39 million
Upper Bound Annual Ingredient Verification Costs	$83 million
Lower Bound Manufacturers Verification Costs	$4 million
Upper Bound Manufactures Verification Costs	$8 million
Annualized Costs (7%, 20 years)	
Lower Bound	$43 million
Upper Bound	$91 million

Table 4. On-Going Testing and Verification Costs shows that these costs would be between $43 million and $91 million per year annualized over 20 years at a seven percent discount rate.

We also recommend that USDA include product reformulation costs for some percentage of the assumed new, non-BE foods. FDA's recent food nutrition labeling rulemaking has estimates of the time

[33] The RIA discussed the same point on pg. 59.

and of the cost to reformulate and to market new food products. FDA has developed a Reformulation Cost Model that USDA can adapt for its revised analysis.

Finally, we recommend USDA re-evaluate the regulation's economic costs in the distribution and in the retail part of the supply chain. USDA's estimates that the regulation will induce an increase in new products equal to 12 percent of existing products and the elimination of a substantial number of existing products would cause substantial logistic costs, marketing costs, and brand destruction. These costs could easily exceed several hundreds of thousands of dollars per new product, meaning around $10 billion in regulatory-induced costs for 95,000 new products.[34] Assuming firms spread these costs over four years, the new product introduction costs could be $800 million per year. Because of the potential magnitude of these costs, USDA should examine the costs of this part of the supply chain more completely.

Costs in Future Years for New and Revised Products

The RIA does not include any costs for new products entering markets in future years. The RIA limits its cost estimate to current products on the market today. After the effective date of the rule, producers must provide the information to consumers and be able to demonstrate compliance for all new or modified products. Ingredient suppliers will also likely face costs as they develop new ingredients in response to changing consumer demand and new technologies. Therefore, the on-going costs for this regulation should include compliance costs for new products.

Recommended Revision

USDA should revise its estimate to include the compliance costs for new products and ingredients introduced into the marketplace. Based on USDA's ERS data, there have been roughly 20,000 new food products introduced in the United States annually in the recent 2010-2016 period.[35] Some of these new products will be exempt from the regulation since they are USDA certified organic or fulfill the meat exemption requirements. For simplicity, we assume that 5,000 new products would be exempt without testing or verification.

For the unit compliance cost for these new products, USDA could extend the existing marketing analysis discussed in the previous section. With the regulation in place, both producers and ingredient suppliers can create new products to sell into the non-BE and the conventional market segment. This knowledge should lower the per unit compliance costs for future products and ingredients. Food processors could source new product ingredients from vendors with available verification information.

As rough estimate, if per product administrative cost is a total of $1,000 throughout the supply chain from ingredient supplier to producer to retailer, the annual costs of the rule increase by $15 million. USDA uses a range of $376 to $3,084 for a low-cost label change; an assumed value of $1,000 is a reasonable estimate for a new product's verification costs. The $15 million is an additional nearly nine percent above USDA's estimated lower bound annualized cost in Scope 3 at a seven percent discount rate.

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[34] We observe suggestive estimates in this range from a variety of sources, providing uncited estimates. For example: https://www.nuvonium.com/blog/view/how-much-will-it-cost-to-market-my-new-product; https://www.linkedin.com/pulse/20141014165748-3775802-from-concept-to-commercialization-10-steps-to-getting-your-food-product-to-market/. We recommend USDA conduct a more detailed estimate in the final rule.

[35] https://www.ers.usda.gov/topics/food-markets-prices/processing-marketing/new-products/. See also RTI, *Cost of Reformulating Foods and Cosmetics Final Report*, prepared for U.S. Food and Drug Administration, RTI Project Number 08184.003, July 2002.

From: Scott Herndon [sherndon@americansugarbeet.org]
Sent: 11/13/2017 4:04:51 PM
To: Hoskins, Dudley - OSEC, Washington, DC [Dudley.Hoskins@osec.usda.gov]
Subject: Disclosure Information
Attachments: Additional information regarding BE testing procedures.pdf; Disclosure Decision Tree.pptx; Disclosure Threshold Scenarios.pdf; U.S. Beet Sugar Industry Comments on Bioengineered Food Disclosure.pdf

Flag: Flag for follow up

Mr. Hoskins,

I wanted to share some information and a corresponding cover letter that the U.S. Beet Sugar industry, the American Farm Bureau Federation, the National Corn Growers Association, Corn Refiners Association, National Milk Producers Federation, the American Soybean Association, National Council of Farmer Cooperatives, and the U.S. Canola Association developed in response to a meeting with Acting Administrator Summers. Mr. Summers, and members of his team, had questions regarding how testing could be conducted to minimize the regulatory burden throughout the value chain. These charts are a recommended framework for implementation of the National Bioengineered Food Disclosure Law.

I have also attached additional research and charts included at the very end of the U.S. Beet Sugar Industry's comments in response to the 30 questions posed by AMS.

Please let me know if you have any questions.

Best,

Scott

Scott Herndon
General Counsel
American Sugarbeet Growers Association
1155 15th Street NW #1100
Washington, DC 20005
202-595-0786

From: Scott Herndon [sherndon@americansugarbeet.org]
Sent: 9/22/2017 3:05:20 PM
To: Summers, Bruce - AMS [Bruce.Summers@ams.usda.gov]; Neal, Arthur - AMS [Arthur.Neal@ams.usda.gov]; May, Laurel - AMS [Laurel.May@ams.usda.gov]; Ohagan, Christopher - AMS [Christopher.Ohagan@ams.usda.gov]
Subject: Thanks and testing cost data
Attachments: Eurofins sampling 2014.pdf; Eurofins Invoice for PCR and ELISA Tests - EUROFINS INVOICE 2014.pdf; Description PCR SOPs isopropanol precipitation - realtime detection 2014.pdf; Description Protein SOPs - ELISA.PDF; SugarTestingbyFactory.pdf

Bruce, Arthur, Laurel, and Chris:

Thanks so much for meeting with me and the other organizations on Wednesday. As requested, I have tracked down the cost information regarding the testing we conducted on beet sugar in 2014 and attached the relevant invoices from Eurofins, the company that conducted the testing. I have attached a summary of the testing results for your review as well.

The first invoice is for the third party gathering of the 69 samples of beet sugar obtained from 23 processing locations across North America ($20,600). The second invoice ($16,215) is for the PCR (DNA) and protein testing that was conducted on all of those samples. I have also attached general descriptions of both types of tests (PCR and protein) obtained from Eurofins. Eurofins thought it would be helpful to let you know for the protein test that "In the 2014 testing cycle I believe we used qualitative Lateral Flow Strips, rather than a full-blown quantitative ELISA, since all the samples were refined sugar. Regardless, Lateral Flow is a similar, antibody-mediated process."

I have spoken to the Executive Vice President of Euofins GeneScan, Inc., Dr. Frank Spiegelhalter, FrankSPIEGELHALTER@eurofinsus.com and the Laboratory Manager, E. Pearce Smith, EdwinPearceSmith@eurofinsus.com. They both would be happy to speak with you to provide more detail. I believe that Dr. Spiegelhalther will be traveling for the next two weeks so Mr. Pearce might be a better immediate contact. He was also the lab manager for the 2014 testing. Full company info is below:

Eurofins GeneScan
2219 Lakeshore Drive, Suite 400
New Orleans, LA 70122
United States
Phone: +1 (504) 297-4330
Fax: +1 (504) 297-4335
Website: www.eurofinsus.com/gmotesting

Please let us know if you have any addition questions as you work through this.

Thanks so much!

Scott

Scott Herndon
Director of Biotechnology and Regulatory Affairs
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DEPARTMENT OF HEALTH AND HUMAN SERVICES

Food and Drug Administration

21 CFR Part 101

[Docket No. FDA–2012–N–1210]

RIN 0910–AF22

Food Labeling: Revision of the Nutrition and Supplement Facts Labels

AGENCY: Food and Drug Administration, HHS.

ACTION: Final rule.

SUMMARY: The Food and Drug Administration (FDA or we) is amending its labeling regulations for conventional foods and dietary supplements to provide updated nutrition information on the label to assist consumers in maintaining healthy dietary practices. The updated information is consistent with current data on the associations between nutrients and chronic diseases, health-related conditions, physiological endpoints, and/or maintaining a healthy dietary pattern that reflects current public health conditions in the United States, and corresponds to new information on consumer understanding and consumption patterns. The final rule updates the list of nutrients that are required or permitted to be declared; provides updated Daily Reference Values and Reference Daily Intake values that are based on current dietary recommendations from consensus reports; amends requirements for foods represented or purported to be specifically for children under the age of 4 years and pregnant and lactating women and establishes nutrient reference values specifically for these population subgroups; and revises the format and appearance of the Nutrition Facts label.

DATES: *Effective date:* The final rule becomes effective on July 26, 2016. *Compliance date:* The compliance date of this final rule is July 26, 2018 for manufacturers with $10 million or more in annual food sales and July 26, 2019 for manufacturers with less than $10 million in annual food sales. See section III, Effective and Compliance Dates, for more detail. The incorporation by reference of certain publications listed in the rule is approved by the Director of the Federal Register as of July 26, 2016.

FOR FURTHER INFORMATION CONTACT: Blakeley Fitzpatrick, Center for Food Safety and Applied Nutrition (HFS–830), Food and Drug Administration, 5100 Paint Branch Pkwy., College Park, MD 20740, 240–402–5429, email: *NutritionProgramStaff@fda.hhs.gov.*

SUPPLEMENTARY INFORMATION:

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folic acid "must be used for purpose of declaration in the labeling of dietary supplements" and "must also be declared in mcg DFE." The comment would revise the footnote to say that folic acid "must be used for foods that contain this nutrient solely in the form of added folic acid. Foods which supply both folate and folic acid must list the predominant form. Folate and folic acid must both be declared in mcg DFE. Additional information regarding the types(s) or sources(s) of the nutrients (*e.g.,* folate, folic acid, or L5–MTHF) and or/relative amounts where more than one form is present, may be included in parentheses." The comment also would revise § 101.9(c)(8)(vii) to require "folate" "for products containing only or predominantly folate" and "folic acid" for "products containing only or predominantly folic acid." (The proposed rule would require, when the amount of folate is declared in the labeling of a conventional food, the use of the name "folate" for products containing either folate alone or a mixture of folate and folic acid and the use of the term "folic acid" when the nutrient is declared in the labeling of a dietary supplement.) The comment also would revise the rule to say that additional information regarding the types(s) or sources(s) of the nutrients (*e.g.,* folate, folic acid, or L-methylfolate) and or/relative amounts where more than one form is present, may be included in parentheses.

(Response) The final rule requires the use of the term "folate" on Supplement Facts labels when folic acid or synthetic folate is added and must be declared and when naturally occurring folate is present and may be declared. The final rule also requires the use of the term "folic acid" in mcg folic acid when folic acid is present. This achieves consistency in terminology between the Supplement Facts and Nutrition Facts labels. If folic acid is declared, manufacturers of dietary supplements must also declare the quantitative amount of folate. The mcg DFE reflects the higher bioavailability of folic acid and certain synthetic folate (*e.g.,* L–5–MTHF) than that of food folate and is the basis of DV.

Under the Supplement Facts label requirements at § 101.36(d), the source ingredient may be identified in parentheses immediately following or indented beneath the name of a dietary ingredient and preceded by the word "as" or "from." When a source ingredient is not identified within the nutrition label, it must be listed in an ingredient statement in accordance with § 101.4(g). However, when a source ingredient is identified in the nutrition label, we do not require it to be listed again in the ingredient statement. With respect to conventional food, the only form that currently can be added to conventional food is folic acid under § 172.345 and not any other forms. If folic acid is added to a conventional food, folic acid must be listed in the ingredient list (§ 101.4(a)).

(Comment 425) Some comments stated that not allowing the term "folate" on dietary supplement labels violates the First Amendment. The comments said we cannot require that labeling to refer to folate as folic acid because, according to the comments, such labeling would then be false.

(Response) The final rule requires the use of the terms "folate" and "folic acid," when declared, on Supplement Facts labels and achieves consistency between the terms used and units of measure in the Supplement Facts and Nutrition Facts labels. Therefore, the comments' First Amendment concerns are no longer applicable.

(Comment 426) One comment said that there is sufficient theoretical and circumstantial evidence that could compel the informed consumer to seek dietary supplements containing methyl folate rather than folic acid. Other comments suggested putting the term "folate" on conventional foods and dietary supplement labels, and using "folic acid" on dietary supplement labels with the source in parentheses (*e.g.,* Folic acid as calcium l-5 methyltetrahyrofolate).

(Response) Under the Supplement Facts label requirements at § 101.36(d), the source ingredient may be identified in parentheses immediately following or indented beneath the name of a dietary ingredient and preceded by the word "as" or "from" (*e.g.,* "folate (as L–5–MTHF-calcium))." When a source ingredient is not identified within the Nutrition Facts label, it must be listed in an ingredient statement in accordance with § 101.4(g). However, when a source ingredient is identified in the Nutrition Facts label, it will not be listed again in the ingredient statement. For conventional food, under § 172.345, the only form that currently can be added to conventional food is folic acid and not any other forms. If folic acid is added to a conventional food, folic acid must be listed in the ingredient list (§ 101.4(a)).

(Comment 427) One comment stated that it is reasonable not to permit the term folate to be used alone on dietary supplement labels because it is not sufficiently specific. The comment added that if DFE is used for foods, it should be used for dietary supplements as well, but that correct calculation is uncertain. The comment suggested using the term FAE (folic acid equivalent) instead of DFE because FAE is based on a well-defined compound, unlike folate naturally present in unspecified food. Furthermore, the comment said, when the folic acid dose is sufficiently small, the biological availability is much better defined than folate from unspecified food. The calculation of FAE would include contribution from all folates, which would include folic acid and L–5–MTHF salts. The comment also stated that, as understanding of folate naturally occurring in food improved, the calculation of its contribution to FAE can be improved.

(Response) We address the requirements for labeling folate in our response to comment 413.

We disagree that the term FAE should be used on the label instead of DFE. Based on the IOM report (IOM 1998), the correct terminology that is accepted by the scientific community is mcg DFE and not FAE. We will, however, monitor the science in this area and, if there are any major changes based on the future consensus report, we will consider whether further changes are needed.

(Comment 428) One comment stated that, while there is consensus that pure folic acid is more bioavailable than naturally occurring folate in food, there is currently no scientific consensus as to the magnitude of this effect. The comment said that one recent review states that the bioavailability of food folate is commonly estimated at 50 percent of folic acid bioavailability, but said this should be considered a rough estimate because the data on the bioavailability of food folate vary between 30 and 98 percent. The comment noted that, even if a dietary supplement's direction for use specifies taking the products with food or alone, many consumers may not comply. The comment also stated that the more precise estimates (*i.e.,* based on consumption of the nutrient in fortified food or a supplement taken with food vs. supplement taken alone) are not justified by the available data. The comment said that our proposed definition, based on IOM recommendations dating to 1998, no longer represents current knowledge and developments in the formulation of foods and supplements accurately. The comment would revise the definition to assign a value to naturally occurring folate at 50 percent of the value of folic acid (as well as at 50 percent of the value of L–MTHF salts on the equimolar basis to folic acid.

The comment also would revise footnote 4 in § 101.9(c)(8)(iv). As

proposed, the footnote would explain that DFE stands for "Dietary folate equivalents" and that 1 DFE equals 1 microgram food folate and equals 0.6 micrograms folic acid from fortified food or as a supplement consumed with food equals 0.5 micrograms of a supplement. The comment would revise the footnote to capitalize the first letters in "folate equivalents" and to state that "1 DFE = 1 mcg naturally occurring folate = 0.5 mcg folic acid (anhydrous basis)* = 0.56 mcg of L-methylfolate calcium salt (anhydrous basis, molecular weight of 497.5))* = 0.93 mcg L-methylfolate glucosamine salt (anhydrous basis, molecular weight of 817.8))*. With respect to the asterisks, the comment said that, because these numbers will often be calculated rather than determined through testing, it is important to specify how water present in the ingredient is to be accounted for in the calculation.

(Response) We disagree that we should assign the value of naturally occurring folate at 50 percent of the value of folic acid (folic acid multiply by 2 instead of 1.7). We agree that the bioavailability of food folate at 50 percent of the bioavailability of folic acid is considered a rough estimate, as data on the bioavailability of food folate may vary between 30 percent and 98 percent. While we recognize that the IOM recommendation dates to 1998, it remains the best scientific consensus report that is available now. We will monitor the science in this area and, if there are any changes based on the future consensus report, we will consider whether to make modifications.

In regard to taking into account the weights of the salts in the formula weights of the available 5–MTHF derivatives, label values and requirements are presented on labels on a weight basis (*e.g.,* mg of calcium, rather than molar equivalents of calcium). Manufacturers are responsible for calculating amounts of the salt forms that, when added, will provide accurate amounts of folate for the label declaration. This is routinely done with other compounds such as minerals (*e.g.,* for calcium, the label states the amount of calcium, not the amount of calcium carbonate that is added).

As for the footnote pertaining to DFE in § 101.9(c)(8)(iv), we have revised it to read as follows: "DFE = Dietary Folate Equivalents; 1 DFE = 1 mcg naturally occurring folate = 0.6 mcg folic acid."

4. Vitamins A, D, and E

Our preexisting regulations, at §§ 101.9(c)(8)(iv) and 101.36(b)(2)(ii)(B), require the use of International Units (IUs) for the labeling of vitamins A, D, and E on the Nutrition and Supplements Facts labels. The preamble to the proposed rule (79 FR 11879 at 11932) described how changes in our understanding of vitamin activity, along with the IOM Labeling Committee's recommendation to change the units of measure for these nutrients to be consistent with the units in the new DRI reports, led us to propose amending § 101.9(c)(8)(iv) to replace IUs for the RDIs for vitamin A, vitamin D, and vitamin E with mcg RAE for vitamin A, mcg for vitamin D, and mg α-tocopherol for vitamin E.

a. General comments.

(Comment 429) Several comments supported changing the units of measure for vitamin A, vitamin D, and vitamin E. One comment supported using mg because, the comment asserted, that is how most registered dietitians give recommendations. Another comment cited a study that reported that physicians typically prescribe vitamin and mineral intakes in mg (Ref. 239). Other comments asked us to retain IUs rather than change to mcg RAE, mcg vitamin D, and mg vitamin E. The comments said that consumers are familiar with IUs and would be confused by use of new units for these nutrients. Other comments seeking to retain IUs as the unit of measure for vitamin D noted that IUs are used on dietary supplements and by clinicians. Another comment requested that the unit of measure for vitamin D be consistent for foods and supplements. One comment supporting the continued use of IUs as a unit of measure noted that the IOM uses IUs for vitamin D.

Other comments recommended that we develop an educational campaign to help consumers understand that changes in the units of measure. Some comments suggested that we make a gradual transition to the new units of measure, including a period during which the labels could use IUs in addition to the new units of measure to help consumer understanding.

(Response) We acknowledge that consumers may need some time to adjust to the new units and consider educational activities important to assist consumers to understand the changes made. However, unlike for vitamins A and E, we have further considered the use of IUs for vitamin D and have determined there are good reasons, specific to vitamin D, to permit the voluntary labeling in IUs for vitamin D in addition to requiring the new mcg units. First, although the IOM Labeling Report (Ref. 25) recommended the use of mcg as the unit of measure for vitamin D, some other IOM materials such as the IOM report on calcium and vitamin D (Ref. 200) present both IUs and mcg as the unit of measure. Thus, we agree, in part, with the comment noting that the IOM uses IUs as the unit of measure. Second, we found that the majority of the U.S. population has usual intakes of vitamin D below the EAR from conventional foods alone, and even when combined with dietary supplements (79 FR 11879 at 11922). Moreover, certain segments of the U.S. population are at risk for inadequacy and may be at increased risk of deficiency. Inadequate intakes of vitamin D are associated with osteoporosis and osteopenia (id.). Third, there are not a wide variety of food sources of vitamin D (79 FR 11879 at 11921), and many individuals rely on vitamin D supplements labeled in IUs to achieve an optimal intake, often on the advice and prescription of a clinician. For these reasons, we have determined it is appropriate to permit the voluntary labeling of vitamin D in IUs, in parentheses, alongside the mandatory declaration in mcg units. In this way, the manufacturer can determine whether to include IUs on the label for its products, based on the use of the product and consumers who may be relying on the advice of a clinician who recommends or prescribes vitamin D in IUs alone, or combined with, mcg units. The reasons we provide for the need for voluntary labeling of IUs for vitamin D are not present with respect to vitamin A or E as the IOM is consistent in presenting units of measure for these nutrients and we have determined them not to be nutrients of public health significance. Therefore, we are replacing IUs with mcg which will be consistent with the IOM Labeling Committee's recommendation that the units of measure be consistent with the DRIs. We agree that the unit of measure for vitamin D should be consistent for foods and supplements. We note that the Supplement Facts label reflects the unit of measure for vitamin D required by §§ 101.9(c)(8)(iv) and 101.36(b)(2)(ii)(B) thus will reflect mcg as the unit of measure for both conventional foods and dietary supplements.

Furthermore, we provide for voluntary labeling of vitamin D in IUs on both conventional food and dietary supplements. Because we have determined that vitamin D is a nutrient of public health significance, we consider that voluntary labeling in IUs for vitamin D will assist consumers in maintaining healthy dietary practices. The voluntary listing of the amount of vitamin D in IUs should be listed in

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parentheses next to the mcg amount for vitamin D.

As for a transition period to the new units of measure, we note that the final rule has a compliance date of July 26, 2018, although the compliance date for manufacturers with less than $10 million in annual food sales is July 26, 2019. This should give manufacturers and consumers some time to convert to the new units of measure and also give us some time to educate consumers about the change.

(Comment 430) Some comments urged that we use the symbol 'µg' instead of 'mcg'.

(Response) We decline to amend the rule as suggested by the comment. While the abbreviation "µg" may also be used for micrograms, the use of "mcg' instead of "µg" may prevent consumers from misinterpreting the prefix µ as m (milli).

b. Specific comments on the units of measure for individual vitamins. Several comments focused on the units of measure for individual vitamins.

(Comment 431) We proposed to change the units of measure for vitamin A in § 101.9(c)(8)(iv) by replacing "IU" with "mcg," representing mcg Retinol Activity Equivalents (RAE). The preamble to the proposed rule explained that the IU for vitamin A does not reflect the carotene:retinol equivalency ratio, that the vitamin A activity of provitamin A carotenoids (such as β-carotene) is less than pre-formed vitamin A (retinol), and that RAEs consider 6 mcg of dietary β-carotene to be equivalent to 1 mcg of purified β-carotene in supplements (79 FR 11879 at 11932). We proposed a similar change dietary supplements in proposed § 101.36(b)(2)(i)(B)(3).

Several comments agreed with the change to mcg RAE. However, other comments opposed changing IUs to mcg RAE; the comments said that the change fails to distinguish between synthetic β-carotene and naturally derived β-carotene in foods and supplements and results in less vitamin A declared on supplements.

One comment noted that we provided only RAE conversions for retinol, beta-carotene, alpha-carotene and beta-cryptoxanthin and said it would be incorrect to apply the same conversion factor to naturally occurring, as compared to synthetically derived, β-carotene.

(Response) We agree there is a difference in biological activity between synthetic and naturally derived β-carotene. Information presented in Table 2 of the proposed rule (79 FR 11879 at 11931) inadvertently omitted a conversion for RAE from β-carotene from supplements. The table in § 101.9(c)(8)(iv) of the final rule includes the conversions for mcg RAE to mcg supplemental β-carotene:

1 retinol activity equivalent (mcg RAE) = 1 mcg retinol
2 mcg supplemental β-carotene
12 mcg of dietary β-carotene
24 mcg of other dietary provitamin A carotenoids (α-carotene or β-cryptoxanthin)

(Comment 432) The proposed rule, at § 101.9(c)(8)(iv), would change the units of measure for vitamin E by replacing "IU" with "mg," representing mg α-tocopherol. The preamble to the proposed rule (79 FR 11879 at 11932) explained that the new measure of vitamin E activity would account for the difference in activity between naturally occurring and synthetic vitamin E.

Several comments supported the definition of vitamin E as mg α-tocopherol. However, other comments disagreed with mg α-tocopherol and recommended that we include other forms, in addition to α-tocopherol, in the definition of vitamin E. The comments said that other forms of vitamin E have biological activity and that some forms are linked to cancer, stroke, and neurodegeneration. One comment cited several studies to support the assertion that other forms of vitamin E have bioactivities that are important to disease prevention and/or therapy (Refs. 240–245). One comment disagreed with the use of mg α-tocopherol for vitamin E and suggested we include different forms of vitamin E and relative amounts so that the vitamin E declaration is not misleading.

(Response) We decline to include other forms in the definition of vitamin E. As we noted in the preamble to the proposed rule (79 FR 11879 at 11926), RDIs for vitamins and minerals are based on the DRIs set by the IOM that reflect the most current science regarding nutrient requirements. The RDA for vitamin E was established for mg of α-tocopherol because α-tocopherol is the only form of vitamin E that is maintained in blood and has biological activity (79 FR 11879 at 11933). We acknowledge the studies submitted to support the assertion that other forms of vitamin E, such as gamma-tocopherol, have biological activity that may be pertinent to disease prevention and/or therapy. However, these individual studies measured outcomes other than induced human vitamin E deficiency assessed by the correlation between red blood cell lysis and plasma α-tocopherol on which the RDA was based (Ref. 246). Jiang *et al.* 2003 studied gamma tocopherol and its metabolite on markers of inflammation in rats (Ref. 241). Mahabir *et al.* 2008 studied the associations between 4 tocopherols (α-, β-, c-, and d-tocopherol) in human diets and lung cancer risk (Ref. 243). The review article by Wolf discussed the biochemical mechanism by which α-tocopherol influences gamma-tocopherol (Ref. 245). Christen et al. 1997 studied the effects of gamma-tocopherol on lipid peroxidation in vitro (Ref. 240). Jiang et al. 2008 studied the effect of different forms of vitamin E and their metabolites on enzyme reactions involved in the inflammation pathway (cyclooxygenase-catalyzed reactions) in vitro (Ref. 242). The review article by Sen *et al.* 2007 discussed tocotrienols and their biological functions. While these animal studies and review articles may suggest biological activity of other forms of vitamin E, outcomes in humans are lacking, thus a totality of evidence for a role of other forms of vitamin E in human health is lacking (Ref. 246). We consider the totality of evidence, such as what is presented in consensus reports like those issued by the IOM, rather than individual studies, to establish the RDIs. Therefore, based on the information provided in the comment, we do not have a basis to include other forms of vitamin E in our definition.

We note, however, that other forms of vitamin E can be listed in the ingredient statement for foods.

(Comment 433) The proposed rule, at § 101.9(g)(10), would require manufacturers to verify the declared amount of both *all rac*-α-tocopherol acetate and RRR-α-tocopherol in the finished food product. The preamble to the proposed rule (79 FR 11879 at 11933) explained that the RDA for vitamin E is 15 mg/day of α-tocopherol and that α-tocopherol is the only form of vitamin E that is maintained in blood and has biological activity. The preamble to the proposed rule also explained that there are eight stereoisomers of α-tocopherol (RRR, RSR, RRS, RSS, SRR, SSR, SRS, SSS) and that only RRR α-tocopherol occurs naturally in foods. Commercially available vitamin E that is used to fortify foods and used in dietary supplements contains esters of either the natural RRR- or, more commonly, mixtures of the 8 stereoisomers (*e.g., all rac*-α-tocopherol acetate). Four stereoisomers (SRR, SSR, SRS, and SSS) are not maintained in human plasma or tissues, so we proposed to limit the new RDA for vitamin E to the four 2R stereoisomeric forms (RRR, RSR, RRS and RSS) of α-tocopherol. We stated that these four forms of α-tocopherol are found in nonfortified and fortified

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conventional foods and dietary supplements and that the *all rac*-α-tocopherol acetate in fortified foods or dietary supplements has one-half the activity of RRR-α-tocopherol naturally found in foods or the 2R stereoisomeric forms of α-tocopherol (id.). However, because AOAC methods cannot individually measure the naturally occurring and synthetic forms of vitamin E, it is necessary to know the amount of both RRR-α-tocopherol and *all rac*-α-tocopherol in a food product to calculate vitamin E activity for declaration as mg α-tocopherol.

One comment suggested that it is more practical for manufacturers of vitamin E esters to ascertain the RRR, RSR, RRS and RSS content in their ingredients and to disclose this information to finished food manufacturers for use in calculating the declared amount of vitamin E, instead of requiring finished food manufacturer to test the finished product to verify the amounts of various forms of vitamin E, especially since valid methods for many food matrices may not be available. The comment was concerned that, even if they can be identified, analytical methods may not be valid for a wide variety of food matrices and may be prohibitively expensive.

Another comment asked that we affirmatively state that, if appropriate new methods become available to distinguish natural and synthetic vitamin E, manufacturers must declare the amount of vitamin E by appropriate and reliable analytical testing.

Another comment disagreed with narrowing the definition of vitamin E to four stereoisomers and said it is burdensome to confirm which stereoisomer is present in synthetic vitamin E additives compared to simply confirming that the additive is, indeed, vitamin E.

(Response) We decline to revise the rule as suggested by the comments.

However, on our own initiative, we are correcting an inadvertent error that we made in the proposed rule. The proposed rule used the term ''*all rac*-α-tocopherol acetate'' when referring to the synthetic form of vitamin E in fortified foods or dietary supplements because esters of synthetic vitamin E are commonly used in fortified foods and dietary supplements. However, the correct term for synthetic vitamin E is *all rac*-α-tocopherol, just as the term for naturally occurring vitamin is *RRR*-α-tocopherol. Esters of synthetic vitamin E are not limited only to ''*all rac*-α-tocopherol acetate'' and also include ''*all rac*-α-tocopheryl succinate.'' We note that the term '*all rac*-α-tocopherol' is the correct term to refer to the synthetic form of vitamin E.

With respect to analytical testing, we decline to speculate on the methods that manufacturers may deem practical to verify the declared amount of both RRR-α-tocopherol and *all rac*-α-tocopherol in finished food products. We acknowledge that it is a new requirement to verify the amount of both RRR-α-tocopherol in the finished food and *all rac*-α-tocopherol added to the food in finished food products when a mixture of both are present in a food. However, without AOAC methods to individually measure these two forms of vitamin E and the inability to determine the amount of RRR-α-tocopherol in a food by subtracting the amount of *all rac*-α-tocopherol from the total amount declared, we need to rely on recordkeeping to verify the amount of vitamin E in a product.

As for the comment's statement that analytical methods may be prohibitively expensive, the practicality or feasibility of using new analytical methods can depend on a variety of factors. For example, a method that uses equipment or technology that is readily available may be less costly compared to a method that uses proprietary equipment or technology. The number of facilities that can use a new analytical method may influence cost. For example, if a large number of facilities are able to use a new analytical method, then testing costs between facilities may become competitive; in contrast, if there are few facilities that can use the analytical method, then testing costs may be less sensitive to competition. Consequently, because we do not know what new analytical methods may exist in the future or the market for those new methods, we cannot say whether those methods will be prohibitively expensive.

We also decline to revise the rule to affirmatively state that manufacturers declare the amounts of vitamin E by appropriate and reliable analytical testing, if appropriate new methods become available. The comment did not explain how manufacturers would be able to determine whether a new method was ''appropriate'' or ''available'' or how differences in opinion as to whether a particular method is ''appropriate'' or ''available'' might be resolved. Current AOAC methods cannot individually measure naturally occurring vitamin E (RRR-α-tocopherol) and synthetic vitamin E (*all rac*-α-tocopherol and its esters) in food products. Nevertheless, we will continue to monitor developments regarding methods to distinguish natural and synthetic vitamin E.

As for the comment objecting to narrowing the definition of vitamin E to four stereoisomers because it is burdensome to confirm which stereoisomer is present in synthetic vitamin E additives, we point out that providing information that a vitamin E additive is only present in a product (rather than confirming the stereoisomers present in the synthetic vitamin E additive) would provide an inaccurate estimation of the vitamin E activity in the body. We reiterate that the RDI for vitamin E is based on the RDA for vitamin E which is limited to the four 2R stereoisomeric forms (RRR, RSR, RRS, and RSS) of α-tocopherol (79 FR 11879 at 11926). Because synthetic vitamin E, also referred to as *all rac*-α-tocopherol, contains both 2R- and 2S-stereoisomers of α-tocopherol and has one-half the activity of the RRR-α-tocopherol naturally found in foods or the other 2R stereoisomers of α-tocopherol, it is necessary to determine the stereoisomers present in a food to determine vitamin E activity.

(Comment 434) One comment noted that the proposed rule did not mention other esters of both natural (d-α-tocopheryl acetate) and synthetic forms of vitamin E (α-tocopheryl succinate) and said we should revise the rule to include these forms.

(Response) We agree that the ester forms of natural and synthetic vitamin E are considered as α-tocopherol forms of vitamin E. The RDA for α-tocopherol is limited to RRR-α-tocopherol (historically and incorrectly labeled d-α-tocopherol) the only form of α-tocopherol that occurs naturally in foods, and the other 2R-stereoisomeric forms of α-tocopherol (RSR-, RRS-, and RSS-α-tocopherol) that are synthesized chemically and found in fortified foods and supplements. Vitamin E compounds include RRR-α-tocopherol (also referred to as d-α-tocopherol or natural) and its esters (*i.e.* RRR-α -tocopheryl acetate, RRR-α -tocopheryl succinate) and *all rac*-α-tocopherol (also referred to as dl-α-tocopherol) and its esters (*i.e., all rac*-α-tocopheryl acetate, *all rac*-α-tocopheryol succinate) (Ref. 247). We note that all of these vitamin E compounds may be present in fortified foods and multivitamins. We have revised the rule to include the ester forms of natural and synthetic vitamin E.

(Comment 435) Another comment requested we provide a conversion in the final rule stating 1 mg α-tocopherol (label claim) = 1 mg RRR-α-tocopherol; 1 mg α-tocopherol (label claim) = 2 mg *all rac*-α-tocopherol.

(Response) We agree with the comment. The final rule provides this

conversion as a footnote in the table in § 101.9(c)(8)(iv): 1 mg α-tocopherol (label claim) = 1 mg α-tocopherol = 1 mg RRR- α-tocopherol = 2 mg *all rac-* α-tocopherol.

(Comment 436) Some comments objected to changing the units of measure for vitamin E. Several comments stated that there are no AOAC international official methods to distinguish between different forms of vitamin E in foods and supplements. One comment objected the change to mg α-tocopherol and said there is a lack of scientifically validated methods capable of individually measuring *all rac*-α-tocopherol acetate and RRR-α-tocopherol.

Another comment said that it is not possible to measure total vitamin E by subtracting *all rac*-α-tocopherol acetate from total vitamin E to determine RRR-α-tocopherol.

(Response) We agree that current AOAC methods cannot individually measure naturally occurring vitamin E (RRR-α-tocopherol) and *all rac*-α-tocopherol in foods. We also agree that it is not possible to measure total vitamin E by subtracting *all rac*-α-tocopherol from total vitamin E to determine RRR-α-tocopherol. For this reason, the final rule, at § 101.9(g)(10)(vi), requires manufacturers to make and keep written records of the amount of *all rac-* α-tocopherol added to the food and RRR-α-tocopherol in the finished food.

We disagree with the comment objecting to changing the unit of measure to mg α-tocopherol because there is a lack of scientifically validated methods capable of individually measuring *all rac*-α-tocopherol and RRR-α-tocopherol. We consider the DRIs that reflect the most current science regarding nutrient requirements as the basis for establishing RDIs and, therefore, the declaration of vitamin E as mg α-tocopherol. The choice of unit of measure for vitamin E is not based on the availability of scientifically validated methods capable of individually measuring *all rac*-α-tocopherol and RRR-α-tocopherol.

5. Niacin

(Comment 437) Our preexisting regulations, at § 101.9(c)(8)(iv), state that the RDI for niacin is 20 mg. The proposed rule would amend § 101.9(c)(8)(iv), in relevant part, by changing the unit of measure from ''mg'' to ''milligrams NE'' where ''NE'' would stand for ''niacin equivalents,'' and a footnote to proposed § 101.9(c)(8)(iv) would explain that 1 milligram NE is equal to 1 milligram niacin or also equal to 60 milligrams of tryptophan. The preamble to the proposed rule discussed updating the RDIs for various nutrients (including niacin) and compared the current RDI of 20 mg against the proposed RDI of 16 mg NE (79 FR 11879 at 11927, 11931).

Several comments supported changing ''mg'' niacin to mg niacin equivalents (NE). The comments said the change would be consistent with the IOM's use of RDAs as the basis for establishing reference values for purposes of food labeling. Another comment referred to the footnote in proposed § 101.9(c)(8)(iv) and noted that ''milligrams NE'' is different from the existing regulation's use of ''milligrams.'' The comment said that it assumed that compliance would be determined by testing the product using AOAC methods for both niacin and tryptophan and that this, if correct, would increase the burden on manufacturers because it will necessitate additional testing.

In contrast, other comments would have us continue to use milligrams as the unit of measure for niacin.

(Response) The RDA for niacin is expressed as niacin equivalents (NE) because the body's niacin requirement is met not only by preformed niacin (nicotinamide, nicotinic acid, and its derivatives) in the diet, but also from conversion from dietary protein containing tryptophan (Ref. 248).

We agree with the comment that compliance with a voluntary declaration of niacin would be determined by analysis, using AOAC methods, for both niacin and tryptophan, or by reference to existing databases for both nutrients. Niacin equivalents would be calculated using the following conversion: NE (niacin equivalents): 1 mg NE = 1 mg preformed niacin = 60 milligrams of tryptophan. While the unit of measurement for the RDI for niacin is listed as mg NE in § 101.9(c)(8)(iv), only the amount ''mg'' will continue to be declared on nutrition and supplement facts labeling.

(Comment 438) One comment asked how compliance will be determined and asked us to clarify whether a declaration of niacin content will be required for products that contain no actual niacin. The comment would revise the rule to include a provision specifying that products containing more than 19 mg of tryptophan (corresponding to 0.32 mg of niacin or 2 percent of the RDI) must declare niacin even if there is no actual niacin present or else the manufacturers of such products might not notice the revised requirements for niacin declaration. Another comment noted that, for many protein-containing products for which there is presently no information on tryptophan required, manufacturers would be required to determine niacin and tryptophan content, either through analytic testing or existing databases.

(Response) The declaration of niacin is voluntary unless it is added as a nutrient supplement to the food or if the label makes a nutrition claim about it. Compliance may be determined by measuring niacin and tryptophan separately. The unit of measure (mg NE) includes both preformed niacin (from nicotinic acid and nicotinamide in the diet or niacin) and niacin resulting from the conversion of tryptophan (Ref. 249), and AOAC methods exist for both niacin and tryptophan. Thus, a declaration of niacin content requires products to include contributions from preformed niacin as well as tryptophan, including those that may not contain preformed niacin.

As for the comment's statement that manufacturers may not notice the revised requirements for niacin declaration, we decline to revise the rule as suggested by the comment. We note that § 101.3(e)(4)(ii) (regarding identity labeling of food in packaged form) states, in relevant part, that a measurable amount of an essential nutrient in a food shall be considered to be 2 percent or more of the Reference Daily Intake (RDI) of any vitamin or mineral listed under § 101.9(c)(8)(iv) per reference amount customarily consumed. We recognize that manufacturers may be unaware of the requirement for niacin declaration in mg and plan to engage in education and outreach explaining the revised changes to units of measurement for vitamins and minerals.

As for the comment that manufacturers would be required to determine niacin and tryptophan content, either through analytic testing or existing databases, we note we have not stated how a company should determine the nutrient content of their product for labeling purposes (Ref. 122). Regardless of its source, a company is responsible for the accuracy and the compliance of the information presented on the label. Use of a database that we have accepted may give manufacturers some assurance in that we have stated that we will work with industry to resolve any compliance problems that might arise for food labeled on the basis of a database that we have accepted. A manual entitled ''FDA Nutrition Labeling Manual: A Guide for Developing and Using Databases'' is available online.

(Comment 439) One comment pointed out that the use of mg NE may not accurately reflect niacin contribution in

foods because the conversion of tryptophan to niacin is highly variable among individuals and because the body uses tryptophan primarily for its role in protein synthesis instead of niacin production. The comment said that using mg NE as the unit of measure could represent an over-estimate of niacin intake in the diet. Another comment was concerned there could be an extra step in food labeling and another potential source of error.

(Response) We disagree that using mg NE may lead to overestimates of niacin intake from foods. We acknowledge that the conversion of tryptophan to niacin may vary among individuals and that tryptophan has a role in protein synthesis. The conversion factor of 1 mg NE = 60 mg tryptophan is the mean of a wide range of individual values from human studies that measured the conversion of tryptophan to urinary niacin metabolites (Ref. 248).

We acknowledge the concern that using mg NE involves an added step of measuring tryptophan, but note that tryptophan is converted to niacin by the body and using mg NE provides a more accurate estimation of available niacin in the body compared to mg of niacin.

(Comment 440) The proposed rule, at § 101.9(c)(8)(iv), would include a footnote stating that ''NE'' means niacin equivalents and that ''1 milligram niacin = 60 milligrams of tryptophan.'' One comment suggested that, for additional clarity and consistency, we should revise footnote 2 to say ''NE = Niacin equivalents, 1 NE = 1 milligram niacin = 60 milligrams of tryptophan.''

(Response) We agree with the comment and have revised the footnote for NE as follows: NE = Niacin equivalents, 1 mg NE = 1 mg niacin = 60 milligrams tryptophan.''

O. Labeling of Foods for Infants, Young Children, and Pregnant or Lactating Women

In the preamble to the proposed rule (79 FR 11879 at 11933), we explained that our general labeling requirements for foods in § 101.9(c) apply to foods for infants, young children, and pregnant and lactating women, with certain exceptions. For example, foods, other than infant formula, represented or purported to be specifically for infants and children less than 4 years of age are not permitted to include declarations of percent DV for the following nutrients: Total fat, saturated fat, cholesterol, sodium, potassium, total carbohydrate and dietary fiber (§ 101.9(j)(5)(ii)(A)). As another example, foods, other than infant formula, represented or purported to be specifically for infants and children less than 2 years of age are not permitted to declare calories from fat, calories from saturated fat, saturated fat, polyunsaturated fat, monounsaturated fat and cholesterol on the Nutrition Facts label (§ 101.9(j)(5)(i)).

The preamble to the proposed rule (79 FR 11879 at 11933) also mentioned that our regulations do not include DRVs or RDIs for nutrients, generally, for infants, children under 4 years of age, or pregnant and lactating women, but there are requirements for a DRV for protein for children 4 or more years of age and RDIs for protein for each of the following subpopulations: (1) Children less than 4 years of age; (2) infants; (3) pregnant women; and (4) lactating women (§ 101.9(c)(7)(iii)).

1. Age Range for Infants and Young Children

Our preexisting regulations, at § 101.9(j)(5), use the age ranges ''less than 2 years of age'' and ''less than 4 years of age'' to establish labeling requirements for foods represented or purported to be specifically for infants and young children. The preamble to the proposed rule (79 FR 11879 at 11933 through 11934) stated that comments to our 2007 ANPRM recommended changing the age categories to infants 7 to 12 months and young children 1 through 3 years (13 through 48 months), consistent with the age ranges used in the IOM's age-specific DRI recommendations. In the preamble to the proposed rule (79 FR 11879 at 11933 through 11934), we discussed why we considered it appropriate to adopt the same age categories as those used in the IOM DRIs for infants and children. In brief, we said:

- Our proposed DVs are based on these age-specific DRIs;
- Infants are transitioning to eating solid foods by 7 through 12 months, and there are a number of foods in the marketplace identified for this age group;
- With respect to children 1 through 3 years of age, using the DRI age range would result in infants no longer being the lower end of the age range in the category of infants and children less than 2 years and less than 4 years of age as specified in § 101.9(j)(5);
- Assigning DVs for children 1 through 3 years of age would ensure consistency with the 1 through 3 year toddler age category established for RACCs specified in § 101.12(a)(2); and
- Because the growth velocity in height is most similar for children 1 through 3 years of age, we consider it appropriate to revise the age range to include children of these ages into a single category for food labeling purposes.

Therefore, we proposed to revise the exceptions for requirements for nutrition labeling provided in § 101.9(j)(5)(i) and the exception to the requirement for the format used for nutrient information on food labeling in § 101.9(d)(1) for foods represented or purported to be specifically for infants and children less than 4 years of age. Specifically, we proposed to replace the current category of infants and children less than 4 years with infants 7 through 12 months and children 1 through 3 years of age.

(Comment 441) Several comments supported providing nutrition information for children less than 4 years because, according to the comments, these subgroups have different nutritional needs. Another comment recommended mandatory nutrition labeling for children less than 12 months and children 1 through 3 years. One comment said that we should continue to allow labeling information on foods for infants less than 7 months, such as infant cereals, or, at a minimum, allow such labeling to remain voluntary.

(Response) We agree, in part, with the comments that recommended mandatory nutrition labeling for infants less than 12 months. We decline to revise the age range for infants to infants less than 12 months because using that age range would leave a 1 month gap as the age for children 1 through 3 years represents 13 through 48 months. We also agree that nutrition labeling on foods represented or purported to be for infants less than 7 months old such as infant cereals should continue to be mandatory. We proposed the age category for labeling of infants 7 through 12 months to be consistent with the age ranges used in the IOM's age-specific DRI recommendations as well as current breastfeeding recommendations for the first 6 months of life (79 FR 11933). Optimally, infants should begin eating complementary foods at around 6 months of age (AAP Section on Breastfeeding 2012, WHO Complementary feeding 2010); however, some infants are being introduced to foods and beverages before then (siega-Riz JADA 2010). To ensure that nutrition labeling includes products for infants and allow for flexibility in timing of complementary food, we have amended § 101.9(j)(5)(i) and (ii) to refer only to ''infants'' as infants through 12 months of age rather than infants less than 12 months (as suggested by the comment) or ''infants 7 through 12 months'' of age as we had proposed. (We have made similar edits in § 101.9(c), (c)(7), (c)(8), (d)(1), (e), and (f) to refer to ''infants through 12 months of age.'')

We note that, while nutrition labeling is mandatory for food for children less than 4 years, we are not establishing DVs for infants less than 7 months of age. Therefore, nutrition information on foods purported for infants less than 7 months would not reflect DVs for that age group.

(Comment 442) One comment said that labeling of foods for infants 7 through 12 months and children 1 through 3 years is overdue and important. The comment said, however, that separate labeling for these two ages is not necessary and could be confusing, so the comment recommended that we use a population approach to set single values for 7 months through 3 years.

Another comment noted that the proposed new age range to set labeling requirements for these foods (infants 7 through 12 months and children 1 through 3 years of age) did not take into account the definition of ''young children'' given in different Codex standards (*e.g.,* 074–1981 Rev. 1–2006) whereby ''young children'' are ''persons from the age of more than 12 months up to the age of 3 years (36 months).''

(Response) We disagree with the comment suggesting an age range of 7 months through 3 years of age. Providing one label for infants and children 7 months through 3 years of age is inappropriate because growth and nutrient needs differ for infants through 12 months of age and children 1 through 3 years of age (beginning at the start of the 13th month through the end of 48th month of age). These differences in growth and development between infants and young children are reflected in the age categories established by the IOM (79 FR 11879 at 11933).

As for the comment noting that we did not take into account the definition of ''young children'' used in certain Codex texts, we note that our age range of children 1 through 3 years of age includes ''persons from the age of more than 12 months up to the age of 36 months.'' We also note that our age range aligns with the age specific category used in the IOM's DRI recommendations for the purposes of establishing DRVs and RDIs for this subpopulation. Our purpose of establishing a DRV or RDI for use in nutrition labeling is distinct from a purpose related to defining the age range when infants and young children are fed processed cereal-based complementary foods (CODEX STAN 074–1981, REV.1–2006). Furthermore, while certain Codex standards such as the Standard for Processed Cereal-based Foods for infants and young children (CODEX STAN 074–1981, REV.1–2006) provide minimum and maximum levels for the composition of processed cereal-based complementary foods, we note that the Codex Guidelines on Nutrition Labelling (CAC/GL 2–1985) (Ref. 121) do not provide Nutrient Reference Value—Requirements that are comparable to our proposed DRVs and RDIs for children 1 through 3 years.

(Comment 443) Some comments asked that we require the declaration of cannabinoid content, nutritional values, and/or health risks pertaining to the consumption of tetrahydrocannabinol (THC) and/or marijuana edibles for all consumers, in particular, children under the age of 4 years as well as pregnant and lactating women.

(Response) We decline to revise the rule as suggested by the comment. We note that section 403(q)(2)(A) of the FD&C Act authorizes the inclusion of nutrients on the label or labeling of food for purposes of providing ''information regarding the nutritional value of such food that will assist consumers in maintaining healthy dietary practices.'' General labeling requirements of products containing THC and/or marijuana edibles is outside the scope of this rule. Therefore, we are making no changes in response to this comment.

2. Mandatory Declaration of Calories and Statutorily Required Nutrients

Currently, foods, other than infant formula, represented or purported to be specifically for infants and children less than 4 years must declare statutorily required nutrients, including calories, calories from fat, total fat, saturated fat, cholesterol, sodium, total carbohydrate, sugars, dietary fiber, and protein. For foods, other than infant formula, represented or purported to be for infants and children less than 2 years, the declaration of certain statutorily required nutrients, which include calories from fat, saturated fat, and cholesterol, is not required or permitted (§ 101.9(j)(5)(i)).

a. Declaration of saturated fat and cholesterol. In the preamble to the proposed rule (79 FR 11879 at 11934), we tentatively concluded that, except for the declaration of calories from fat, the declaration of statutorily required nutrients that include saturated fat and cholesterol on the label of foods represented or purported to be specifically for infants 7 through 12 months and children 1 through 3 years of age should be mandatory because: (1) The declaration of calories and these nutrients is mandated by section 403(q) of the FD&C Act, and we have no basis on which to not require or permit their declaration as discussed previously; and (2) these nutrients are essential in fostering growth and maintaining good health during a critical stage of human development and physiology and, therefore, their mandatory declaration can assist in maintaining healthy dietary practices. We proposed to remove § 101.9(j)(5)(i) and revise and redesignate current § 101.9(j)(5)(ii) as § 101.9(j)(5)(i).

Similarly, foods consumed by pregnant and lactating women must declare statutorily required nutrients, including calories, calories from fat, total fat, saturated fat, cholesterol, sodium, total carbohydrate, sugars, dietary fiber, and protein. Women of reproductive age consume the same foods as the general population and, in general, continue consuming similar foods during pregnancy and lactation. In the preamble to the proposed rule (79 FR 11879 at 11934), we tentatively concluded that, except for the declaration of calories from fat, the declaration of statutorily required nutrients should be mandatory because the declaration of calories and these nutrients is mandated by section 403(q) of the FD&C Act and we have no basis on which to not require or permit their declaration as discussed previously. Thus, we proposed to require the mandatory declaration of calories, and the amount of total fat, saturated fat, *trans* fat, cholesterol, sodium, total carbohydrate, dietary fiber, sugars, and protein for foods represented or purported to be specifically for infants 7 through 12 months of age, children 1 through 3 years of age, and pregnant and lactating women, and permit the declaration of calories from saturated fat such that the declaration of these nutrients on foods for these populations would be subject to the same requirements applicable to foods for the general population.

(Comment 444) Several comments supported the declaration of saturated fat and cholesterol on labeling for infants and children 1 through 3 years old and agreed such labeling will help maintain healthful dietary practices. In response to our request for information on whether consumers may be confused by these changes, one comment said that its products have been labeled for children under 2 years as well as for children less than 4 years of age on the market for many years. The comment noted that these dual label formats include the declaration of both saturated fat and cholesterol and the company has received no comments or concerns about the inclusion of this information on its labels from either consumers or health care professionals. The comment said that declaring saturated fat and cholesterol in addition to *trans* fat on infant foods will be more helpful in

food selection than having *trans* fat alone. The comment said declaring saturated fat, cholesterol, and *trans* fat will provide more information on the fat composition of foods and their relationship to chronic disease risk. The comment also noted that some children as young as 12 months, with a family history of obesity, dyslipidemia, or CVD, may benefit from a diet lower in saturated fat and that having saturated fat on food labels can assist families in choosing foods that are lower in saturated fat while maintaining total fat intakes.

Another comment said we should not finalize the rule until we had conducted appropriate research, including consumer testing, to better understand the impacts of declaring saturated fat and cholesterol on the labels of products represented or purported to be specifically for infants and children 1 through 3 years of age and to determine if an explanatory footnote would assist in improving consumer understanding when accompanying any relative declaration. The comment also noted that relevant empirical research is not available to determine whether the declaration of saturated fat and cholesterol will result in restricted intakes for infants and children ages 1 through 3 years old. One comment would revise the rule to include a voluntary footnote stating that ''total fat should not be limited in the diets of children less than 2 years unless directed by a physician'' or similar wording to provide dietary guidance to parents and other caregivers to help assure total fat is not restricted in the diet of young children.

(Response) We acknowledge that products dual labeled for children under 2 and children less than 4 years of age include the declaration of both saturated fat and cholesterol. We agree that declaration of saturated fat and cholesterol provides more nutrition information and can help consumers make informed choices and maintain a healthy diet, and the final rule requires the declaration of saturated fat and cholesterol on Nutrition Facts labeling for infants and children 1 through 3 years of age.

As for the comment regarding consumer testing, we disagree that consumer testing is necessary before we can require the declaration of saturated fat and cholesterol on Nutrition Facts labels for infants and children 1 through 3 years of age. Section 403(q) of the FD&C Act lists total fat, saturated fat, and cholesterol as nutrients required on nutrition labeling. These nutrients are essential for growth and development, thus their mandatory declaration can assist consumers in maintaining healthy dietary practices (79 FR 11879 at 11934). We considered the Integrated Guidelines for Cardiovascular Health and Risk Reduction in Children and Adolescents which suggest a diet with saturated fat less than 10 percent of calories and cholesterol intake less than 300 mg/day can safely and effectively reduce the levels of total and LDL cholesterol in healthy children (Ref. 250). This type of diet may have similar effects when started in infancy and sustained throughout childhood into adolescence (Ref. 250).

We acknowledge, in general, that total fat should not be limited in the diets of young children less than 2 years of age unless directed by a health professional. In response to the comment noting that research is unavailable on whether declaration of saturated fat and cholesterol will result in restricted intakes for infants and children, we intend to monitor fat and cholesterol intakes in these age groups and will consider whether to revisit our requirements for this labeling, as appropriate.

We also decline to include a voluntary footnote. We intend to monitor fat intakes and educate consumers on changes to the labeling of foods for infants through 12 months of age and children 1 through 3 years of age.

b. Percent DV declaration. In the preamble to the proposed rule (79 FR 11879 at 11935), we explained that, under our preexisting regulations, the percent DV declaration is not permitted on the food label for foods, other than infant formula, represented or purported to be specifically for infants and children less than 4 years (which includes infants and children less than 2 years) for total fat, saturated fat, cholesterol, sodium, potassium, total carbohydrate, and dietary fiber (§ 101.9(j)(5)(ii)). Percent DV is required for protein and vitamin A, vitamin C, iron, and calcium. We tentatively concluded that it is appropriate to require declarations of percent DV for those nutrients for which we are establishing a DRV or RDI for infants 7 through 12 months, for children 1 through 3 years of age, and for pregnant and lactating women (except for a % DV for protein for pregnant and lactating women), and this change would be reflected in redesignated § 101.9(j)(5)(i).

(Comment 445) One comment would retain a requirement for the mandatory declaration of percent DV for protein on infant foods.

In contrast, another comment would not require the mandatory declaration of the percent DV for protein on labels of foods for children aged 1 through 3 years. The comment cited dietary intake data suggesting that protein intakes are above 40 grams per day and from high quality sources. Another comment recommended allowing for the use of the PDCAAS for determining the percent DV for protein for all population groups, including infants. The comment asked us to clarify the acceptability of PDCAAS for determining protein quality for foods for infants and specify the specific amino acid pattern that should be used (*i.e.,* IOM pattern) and to reference the pattern by Table number.

(Response) The final rule requires the mandatory declaration of percent DV for protein on foods for infants though 12 months of age and children 1 through 3 years of age. While the evidence suggests that protein intake is adequate and of high quality, the level and quality of protein present in a food remain an important consideration in food selection for infants because infant diets are derived from a limited number of foods. Calculating the percent DV for protein incorporates a measure of protein quality. Thus, the percent DV declaration is a useful tool to indicate protein quality to the consumer. Because of the importance of adequate high quality protein in the diets of infants and young children, we conclude that the percent DV declaration for protein for infants though 12 months of age and children 1 through 3 years of age should remain mandatory.

We disagree with the comment asking that we allow for the use of the PDCAAS to determine protein quality for infants. The PDCAAS allows evaluation of food protein quality based on the needs of humans as it measures the quality of a protein based on the amino acid requirements (adjusted for digestibility) of a 2- to 5-year-old child (considered the most nutritionally demanding age group), not infants (Ref. 251). Protein quality is important during infancy for growth and development. We established the protein efficiency ratio (PER) as the method of determining protein quality (see 79 FR 7934 at 8022) for infants based on recommendations from the 1991 WHO Protein Quality report. A protein source may contain the necessary amino acids, but they may be in a form that an infant cannot digest and absorb. The PER method, unlike chemical measures of protein composition, provides an estimate of the bioavailability or amount absorbed, of the protein.

(Comment 446) One comment said that, if the percent DV for protein remains mandatory, we should provide

an exemption from the mandatory declaration of percent DV for protein for foods intended for infants and children aged 1 through 3 years that declare less than 1 gram of protein per serving, such as fruits, because these foods contain an insignificant amount of protein and are not expected to contribute meaningfully to protein intake. The comment also would revise the rule to allow the optional declaration of ''0% DV'' instead of the phrase ''not a significant source of protein'' on infant foods with a protein quality of less than 40 percent of casein as measured by PER or less than 40 percent by PDCAAS or other comparable method. The comment explained that these options will help save label space, especially on small packages, while still providing meaningful information on protein quantity relative to the DV.

(Response) We decline to revise the rule as suggested by the comment. While we recognize that the protein quantity of some foods, such as fruits, may be small, we consider the mandatory declaration of percent DV to provide important information on protein quality to the consumer. In establishing mandatory declaration of percent DV for protein on foods intended for infants through 12 months of age and children aged 1 through 3 years and associated statements of ''less than 1 g of protein per serving'' or ''not a significant source of protein,'' we considered that: (1) Protein is of critical importance in maintaining good health because it supplies essential amino acids and is a principal source of calories along with fat and carbohydrate; and (2) calculating the percent DV for protein incorporates a measure of protein quality. Thus, the percent DV declaration is a useful tool to indicate protein quality to the consumer.

While label space on small packages may be a concern, we decline to make the change requested by the comment that would allow the optional declaration of ''0% DV'' instead of the phrase ''not a significant source of protein'' on infant foods with a protein quality of less than 40 percent of casein as measured by PER or less than 40 percent by PDCAAS or other comparable method. As explained in part II.I and in our response to comment 445, we concluded that the PDCAAS was the most suitable pattern for use in the evaluation of dietary protein quality for all age groups, except infants through 12 months of age. We established the PER as the method of determining protein quality for infants because infants cannot digest and absorb all forms of protein; thus, PDCAAS or another comparable method that scores the amino acid profile of the specific food protein after it has been digested is not appropriate.

3. Declaration of Non-Statutory Nutrients Other Than Essential Vitamins and Minerals

In the preamble to the proposed rule (79 FR 11879 at 11935), we stated that foods, other than infant formula, represented or purported to be specifically for infants and children less than 2 years of age are not permitted to declare calories from saturated fat and the amount of polyunsaturated fat and monounsaturated fat (§ 101.9(j)(5)(i)), whereas soluble fiber, insoluble fiber, and sugar alcohols can be declared voluntarily. Polyunsaturated fat, monounsaturated fat, soluble fiber, insoluble fiber, and sugar alcohols can be declared voluntarily on the label of foods represented or purported to be specifically for children 2 through 4 years of age, and pregnant and lactating women.

For foods represented or purported to be specifically for children 1 through 3 years of age and pregnant and lactating women, we considered whether to propose the mandatory or voluntary declaration of non-statutory nutrients. In the preamble to the proposed rule (79 FR 11879 at 11935), we said that most advisory consensus and policy reports on which we rely for the general population apply to children 2 years of age and older and pregnant and lactating women, unless noted otherwise (*e.g.,* 2010 DGAC and health claims (§ 101.14(e)(5)).

a. Voluntary declaration of calories from saturated fat, and the amount of polyunsaturated and monounsaturated fat. Our preexisting regulations, at § 101.9(j)(5)(i), state that foods, other than infant formula, represented or purported to be specifically for infants and children less than 2 years of age must bear nutrition labeling with certain, specific exceptions. Among the exceptions, the label is not to include polyunsaturated fat or monounsaturated fat.

The proposed rule would remove the restriction regarding the declaration of polyunsaturated fat and monounsaturated fat on foods represented or purposed to be specifically for children less than 2 years of age. In the preamble to the proposed rule (79 FR 11879 at 11935 through 11936), we explained that, for infants 7 to 12 months, there are no specific recommendations provided about calories from saturated or polyunsaturated or monounsaturated fat. We also stated there is some evidence to suggest that reduction of total and LDL cholesterol levels can occur with reducing saturated fat intake to less than 10 percent of calories, beginning in infancy and sustained throughout childhood into adolescence, that there is no evidence to suggest that infants 7 through 12 months of age would be different than children 1 through 3 years of age, and that there is no basis to continue to provide an exception that does not permit the declaration of calories from saturated fat, or polyunsaturated and monounsaturated fats on foods represented or purported to be specifically for infants and children less than 2 years of age.

(Comment 447) One comment argued the declaration of alpha linoleic acid (ALA) on foods for infants and children 7 months to 3 years of age should be considered for voluntary labeling using the AI as the basis for a DRV. The comment noted that much of the evidence for a health benefit of n-3 fatty acids derives from studies on infants, and labeling of ALA is consistent with FDA's criteria of encouraging health dietary practices. Another comment recommended that we examine NHANES data for ALA consumption to determine whether there is a public health risk from inadequate dietary intake.

(Response) We decline to amend the rule to permit the voluntary labeling of ALA on labels or labeling for foods intended for infants though 12 months of age and children 1 through 3 years of age and to use the AI for ALA to establish a DRV.

We agree with promoting healthy dietary practices in this subpopulation; however, well-established evidence for ALA and disease risk reduction in adulthood and infancy is lacking (Ref. 29). As discussed in part II.F.4, we decided that, because of the lack of well-established evidence for a role of n-3 or n-6 polyunsaturated fatty acids in chronic disease risk and the lack of a quantitative intake recommendation, the declarations of α-linolenic acid as well as other n-3 and n-6 polyunsaturated fatty acids are not necessary to assist consumers to maintain healthy dietary practices. Because the declaration of ALA is not permitted on labeling, a DRV for this nutrient is unnecessary.

We disagree with the analysis of NHANES data for ALA intake to determine public health risk from inadequate dietary intake. An analysis of dietary intake data alone does not meet our criteria of public health significance. Moreover, an analysis of ALA intakes from NHANES data cannot determine inadequacy of dietary intake

because an EAR has not been established for ALA. EARs, not AIs, are used for assessing the statistical probability of adequacy or nutrient intakes of groups of people (79 FR 11879 at 11885).

(Comment 448) One comment noted that we proposed mandatory labeling of the quantitative amount of some nutrients (*trans* fatty acids for which there is no DRI) on foods for infants aged 7 through 12 months and children aged 1 through 3 years. The comment said we should provide for the voluntary declaration of docosahexaenoic acid (DHA) on these foods to encourage healthy dietary practices.

(Response) We decline to revise the rule as suggested by the comment. Our regulations, at § 101.9(c)(2)(ii), require the declaration of *trans* fat on nutrition labeling for people of all ages because the consumption of *trans* fats may affect their risk of CHD; therefore, the presence or absence of *trans* fat in a food product is a material fact that consumers need to know to make healthy choices and allow them to reduce risk of CHD. *Trans* fat continues to be a nutrient with public health significance because of its well-established role in chronic disease through its effect on blood cholesterol levels (79 FR 11879 at 11896). However, DHA lacks well-established evidence for its role in chronic disease as well as growth or neural development (IOM Macro report). As discussed in part II.F, voluntary labeling of DHA is not permitted because of the lack of well-established evidence for DHA's role in chronic disease risk and lack of a quantitative intake recommendation (79 FR 11879 at 11898).

(Comment 449) One comment cited a 2011 IFIC survey suggesting that 45 percent of consumers were already eating foods containing n-3 fatty acids to benefit cognitive development, especially in children and 39 percent were somewhat likely to begin eating n-3 fatty acids for this health benefit in the next 12 months. The comment said that continued allowance of ALA nutrient content claims, absent a voluntary declaration of DHA, increases the likelihood that consumers may purchase foods for a benefit that the food will not supply. The comment also said that allowing polyunsaturated fat labeling of foods for children younger than 2 years without allowance for labeling of individual polyunsaturated fatty acids creates a scenario where polyunsaturated fat values, inflated by ALA, may mislead consumers actually seeking DHA.

(Response) The comments did not provide, and we are not aware of, data or information to support the claim that consumers seeking to consume DHA would be misled by the voluntary declaration of polyunsaturated fats or an ALA nutrient content claim on labeling for children less than 2 years of age. Therefore, we are not making changes in response to this comment.

We acknowledge the 2011 IFIC survey conclusions suggesting that consumers eating foods containing n-3 fatty acids are somewhat likely to begin eating these foods to benefit cognitive development. We also recognize that total polyunsaturated fats in foods include both n-6 and n-3 polyunsaturated fatty acids and the n-3 polyunsaturated fatty acids content may include ALA and DHA.

However, we are unable to determine, based on the information provided in the comment, if some consumers seeking to consume DHA may be confused or misled by the declaration of total polyunsaturated fats or the ALA nutrient content claim. Furthermore, we are unable to determine if consumers understand that ALA may be converted to DHA. Without knowledge of the conversion from ALA to DHA, consumers would not be able to distinguish between the level and type of n-3 fatty acids in the food.

Thus, the final rule removes the restriction regarding the declaration of calories from saturated fat, polyunsaturated fat, and monounsaturated fat on foods represented or purposed to be specifically for infants through 12 months of age and children 1 through 3 years of age.

b. Voluntary declaration of soluble fiber, insoluble fiber, and sugar alcohols. In the preamble to the proposed rule (79 FR 11879 at 11936), we stated that, while quantitative intake recommendations are lacking for soluble fiber, insoluble fiber, and sugar alcohols, there is well-established evidence for the role of these nutrients in chronic disease risk, risk of a health-related or a beneficial physiological endpoint (*i.e.,* CHD, improved laxation, or dental caries). We also said that there is no evidence to suggest that the role of these nutrients would be different among infants 7 through 12 months, children 1 through 3 years of age, or pregnant and lactating women compared to the general population. As a result, we did not propose any changes to the provisions for the voluntary declaration of soluble fiber, insoluble fiber, and sugar alcohols on the label of foods represented or purported to be specifically for infants 7 to 12 months, children 1 through 3 years of age, or pregnant and lactating women.

We did not receive comments on this topic, so no changes to the rule are necessary.

c. Mandatory declaration of trans fat. In the preamble to the proposed rule (79 FR 11879 at 11936), we stated that *trans* fat must be declared on the Nutrition Facts label and that our regulations do not provide exceptions for foods represented or purported to be specifically for infants, young children, or pregnant and lactating women. We noted that cardiovascular disease is known to begin in childhood (id.). Thus, we tentatively concluded that declaration of *trans* fat continues to be necessary to assist consumers in maintaining health dietary practices, including among infants, young children, and pregnant and lactating women, and we did not propose any changes to the mandatory declaration of *trans* fat on the label of foods represented or purported to be specifically for infants, children 1 through 3 years of age, or pregnant and lactating women.

Trans fat declaration is voluntary when the total fat content of a food is less than 0.5 grams (§ 101.9(c)(2)(ii)). In addition, if a manufacturer does not declare the *trans* fat content because total fat amount is less than 0.5 grams, then the statement "Not a significant source of *trans* fat" must be placed at the bottom of the table of nutrient values.

We did not receive comments on this topic and have finalized this provision without change.

d. Mandatory declaration of added sugars. Our preexisting regulations do not provide for the declaration of added sugars on the Nutrition Facts label, but the proposed rule would require the mandatory declaration of added sugars on the Nutrition Facts label. Additionally, in the **Federal Register** of July 27, 2015 (80 FR 44303), we published a supplemental proposed rule that would, among other things, establish a Daily Reference Value (DRV) of 10 percent of total energy intake from added sugars and require the declaration of the percent DV for added sugars on the label.

(Comment 450) Several comments supported mandatory declaration of added sugars. One comment stated that sugar is used as a means to attract children, and this practice should be discouraged.

Another comment opposed the mandatory labeling of added sugars for infants and children aged 1 through 3 years and pregnant and lactating

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women. The comment argued that scientific consensus is lacking for the health effects of added sugars alone versus sugars as a whole and recommended careful consideration of the totality of the scientific evidence, as well as consideration of compliance and other technical issues. The comment also noted that consumer testing is also highly important prior to any determination relative to added sugars being made.

(Response) We disagree that added sugars should not be required on the label for infants and children aged 1 through 3 years and pregnant and lactating women. We discuss in part II.H.3 our rationale for requiring the declaration of added sugars on the label for the general population. We are also basing an added sugars declaration on labeling for infants, children 1 through 3 years of age, pregnant women, and lactating women on the need to provide consumers with information to construct a healthy dietary pattern that meets the dietary recommendations for added sugars.

In response to the comment about the totality of evidence for the health effects of added sugars, we discuss in part II.H.3 that rather than basing a declaration of added sugars on an association with risk of chronic disease, a health-related condition, or a physiological endpoint, we are considering a declaration of added sugars in the context of how it can assist consumers in maintaining healthy dietary practices by providing information to help them limit consumption of added sugars, and to consume a healthy dietary pattern. We have established that there is public health significance of added sugars through other evidence related to a healthy dietary pattern low in sugar-sweetened foods and beverages that is associated with reduced risk of CVD, through consumption data showing that Americans are consuming too many calories from added sugars, through evidence showing that it is difficult to meet nutrient needs within calorie limits if one consumes too many added sugars, and through evidence showing that increased intake of sugar-sweetened beverages is associated with greater adiposity in children.

The comment did not explain what compliance and other technical issues merit further consideration. In response to the comment noting the importance for consumer testing of a declaration of added sugars, we have received several comments on this topic and discuss responses in part II.H.3.g.

While the declaration of added sugars is mandatory, we are not establishing a DRV for added sugars for infants through 12 months. Dietary recommendations for infants through 12 months suggest introducing complementary foods such as infant cereal, vegetables, fruits, meat, and other protein-rich foods modified to a texture appropriate (*e.g.,* strained, pureed, chopped, etc.) for the infant's developmental readiness one at a time. A DRV for added sugars for infants through 12 months is not necessary as the infant diet is comprised primarily of breast milk and/or infant formula as well as complementary foods. As the food introduced does not comprise the majority of the infant diet, a DRV is not necessary to compare added sugars in the context of a daily diet. Mandatory declaration of added sugars for infants through 12 months of age can help consumers limit the added sugars in the limited complementary foods that are being introduced individually.

(Comment 451) One comment would modify the definition of added sugars to exclude ingredients that are inherent in the food or are present for purposes other than sweetening the food and that this modified definition should apply for adults and children between 7 months to 3 years of age, and pregnant and lactating women.

(Response) We received many comments on the definition of added sugars and, in part II.H.3.n, discuss ingredients that are inherent in the food, such as naturally occurring sugars, and the intended purpose of sweetening. The comment did not explain why a regulatory definition for added sugars should be different for infants, children 1 through 3 years of age, and pregnant women, and lactating women, so we decline to revise the rule as suggested by the comment.

e. Voluntary declaration of fluoride. Our preexisting regulations do not provide for the declaration of fluoride on the Nutrition Facts label of any foods. The proposed rule would allow voluntary declaration of fluoride on the labeling of foods for the general population, and we also tentatively concluded that the declaration of fluoride on foods represented or purported to be specifically for children 1 through 3 years of age and pregnant and lactating women can assist in maintaining healthy dietary practices. We stated, in the preamble to the proposed rule (79 FR 11879 at 11937 through 11938), that evidence on dental caries is lacking for infants 7 through 12 months of age, but we did not expect the role of fluoride in the protection against dental caries to be different from other age groups. Therefore, proposed § 101.9(c)(5) would permit the voluntary declaration of fluoride on foods represented or purported to be specifically for infants 7 through 12 months of age, children 1 through 3 years of age, and pregnant and lactating women.

We did not receive comments on this topic and have finalized the provision to permit the voluntary declaration of fluoride on foods represented or purported to be specifically for infants through 12 months, children 1 through 3 years of age, pregnant women, and lactating women.

4. Declaration of Essential Vitamins and Minerals

Our preexisting regulations require the declaration of vitamin A, vitamin C, calcium, and iron on the Nutrition Facts label, and there are no specific exceptions to this requirement for foods represented or purported to be specifically for infants and children less than 2 years and children less than 4 years of age, and pregnant and lactating women. In the preamble to the proposed rule (79 FR 11879 at 11937), we explained that the AIs for essential vitamins and minerals (and RDAs for iron and zinc) for infants 7 through 12 months of age are based on the average intake of nutrients that infants consumed from breast milk, complementary foods, and/or supplements with the understanding that these sources provided sufficient amounts of the nutrients to meet the infant's daily needs. The AIs (as well as the RDAs for iron and zinc) for infants were not based on endpoints related to chronic disease risk, or a health-related conditions or health-related physiology. Furthermore, because the AI represents intakes that are considered adequate and are based on average nutrient intakes from breast milk, foods, and/or supplements, the presence of an AI indicates that there is not a public health concern about adequate intake of that nutrient. So, rather than determine public health significance for a nutrient during infancy based on an AI for infants, we considered the importance of the nutrient in establishing healthy dietary practices during infancy for later in life, as well as the relevant available information for children 1 through 3 months of age that may also be applicable to infants. For nutrients with an RDA for infants 7 through 12 months of age (*i.e.,* iron and zinc), we considered the factors for mandatory and voluntary labeling described in section I.C to determine whether to propose mandatory or voluntary labeling for the nutrient.

For the declaration of essential vitamins and minerals for children 1

through 3 years of age and pregnant and lactating women, we said, in the preamble to the proposed rule (79 FR 11879 at 11937) that we would use the same considerations, based on the same rationale as we set forth and proposed for the general population, because scientific and policy considerations are generally the same and the DGA recommendations apply to Americans 2 years of age and older. We also explained that, while NHANES data were collected in lactating women, we did not include these data in our analysis because the sample size of lactating women was small, and we could not reliably estimate mean intake and status of this population (id.). However, we stated that the conclusions made about nutrient inadequacy during pregnancy are applied to lactating women since the needs of essential vitamin and minerals are increased for both pregnant and lactating women, and we proposed to remove the provision in § 101.9(c)(8)(i) that requires separate declaration of percent DVs based on both RDI values for pregnant women and for lactating women in the labeling of foods represented or purported to be for use by both pregnant and lactating women.

We did not receive comment on this topic and are removing the provision in § 101.9(c)(8)(i) regarding separate declaration of percent DVs based on both RDI values for pregnant women and for lactating women in the labeling of foods represented or purported to be for use by both pregnant and lactating women.

a. Mandatory declaration of calcium and iron. We did not propose any changes to the mandatory declaration of calcium on foods for the general population. In the preamble to the proposed rule (79 FR 11879 at 11937), we stated that the AI for calcium for infants 7 through 12 months of age is based on average calcium consumption of these nutrients, rather than chronic disease risk, health related-condition, or physiological endpoints and that, for children 1 through 3 years of age and pregnant and lactating women, the RDAs for calcium are based, in part, on bone health.

Our analysis of NHANES 2003–2006 data estimated that infants ages 7 to 12 months have usual calcium intakes above the AI and that about 12 percent of children 1 through 3 years of age had usual intakes of calcium below the EAR, based on intakes from conventional foods only (see 79 FR 11879 at 11937). We said, in the preamble to the proposed rule (id.), that promoting the development of eating patterns that are associated with adequate calcium intake later in life is important given that calcium intakes are inadequate for the majority of the population. Intakes of calcium, which is necessary for growth and bone development, are inadequate among children. Similar to the general population, approximately 20 percent of pregnant women consumed less than the EAR for calcium from conventional foods as well as from conventional foods and supplements. Consequently, we tentatively concluded that calcium is a nutrient of public health significance for children 1 through 3 years of age and for pregnant and lactating women and that, because calcium is important for growth and development, calcium is of public health significance for infants 7 through 12 months of age.

With respect to iron, we stated, in the preamble to the proposed rule (id.) that, while the EAR and RDA are based on daily iron requirements and not directly on chronic disease risk, iron deficiency is associated with delayed normal infant motor function (*i.e.,* normal activity and movement) and mental function (*i.e.,* normal thinking and processing skills) and that our analysis of NHANES 2003–2006 data estimated that about 18 percent of infants ages 7 through 12 months have usual iron intakes below the EAR, based on intakes from conventional foods only and 4 percent of infants ages 7 through 12 months have usual iron intakes below the EAR based on intakes from conventional foods and supplements. For children 1 through 3 years of age, about 1 percent of children have usual iron intakes below the EAR, based on intakes from conventional foods only and 0.4 percent of children have usual iron intakes below the EAR based on intakes from conventional foods and supplements. While total iron intakes appear adequate, the prevalence of iron deficiency in children ages 1 to 2 years has been reported to be 14.4 percent and the prevalence of iron deficiency anemia in children younger than 5 years has been reported to be 14.9 percent (see 79 FR 11879 at 11937). We also stated that inadequate iron intakes during pregnancy are of public health significance because of the adverse effects for both the mother and the fetus (such as maternal anemia, premature delivery, low birth weight, and increased perinatal infant mortality) and that our analysis of data collected by NHANES 2003–2006 estimated that 5 percent of pregnant women 14 to 50 years of age had usual iron intakes below the EAR based on intakes from conventional foods and 4 percent of pregnant women 14 to 50 years of age had usual iron intakes below the EAR based on intakes from conventional foods and supplements (see 79 FR 11879 at 11937). Among pregnant women aged 12 to 49 years, 25 percent were iron deficient and 13 percent had iron deficiency anemia. While intakes appear adequate for most individuals, the prevalence of iron deficiency and iron deficiency anemia indicates that iron deficiency is of public health significance for pregnant women. Therefore, we tentatively concluded that iron is a nutrient of public health significance for lactating women as well.

Thus, we proposed to amend § 101.9(c)(8)(ii) to require the mandatory declaration of calcium and iron on foods represented or purported to be specifically for infants 7 to 12 months, children 1 through 3 years of age, or pregnant and lactating women.

We did not receive any comments with respect to mandatory declaration of calcium and iron for these populations, and so, other than replacing "infants 7 to 12 months" with "infants through 12 months," we have finalized the provisions without change.

b. Mandatory declaration of vitamin D and potassium. We proposed to require the declaration of vitamin D on foods for the general population. With respect to infants, we stated, in the preamble to the proposed rule (79 FR 11879 at 11938), that the AI for vitamin D for infants was based on maintenance of serum 25(OH)D concentrations at a level to achieve and maintain serum 25(OH)D concentrations above a defined level (30 to 50 nmol/L) in order to meet the needs of the majority of the infants and support bone accretion and that DRIs (EAR and RDA) for vitamin D were established at a level to achieve and maintain serum 25(OH)D concentrations above a defined level (40 to 50 nmol/L) to maintain bone health for children 1 through 3 years of age and pregnant women. Although serum 25(OH)D data were not available in NHANES 2003–2006 for infants ages 7 to 12 months, we noted that our analysis of NHANES 2003–2006 dietary data showed that 28.7 and 33.6 percent of infants ages 7 to 12 months have usual vitamin D intakes above the AI from conventional foods and conventional foods plus supplements, respectively (see 79 FR 11879 at 11938).

Our analysis of NHANES 2003–2006 data showed that about 3 percent of children 1 through 3 years of age had serum 25(OH)D levels below 40 nmol/L, while an analysis of NHANES 2005–2008 dietary data showed that, assuming minimal sun exposure, about 82 percent of these children had usual vitamin D intakes below the EAR from

conventional foods only and 66 percent had usual intakes below the EAR from conventional foods and supplements (see 79 FR 11879 at 11938). For pregnant women, 15 percent had serum 25(OH)D levels below 40 nmol/L, while about 88 percent of pregnant women had usual vitamin D intakes below the EAR from conventional foods only and 48 percent had usual intakes below the EAR from conventional foods and supplements (id.). We tentatively concluded that vitamin D has public health significance in children 1 through 3 years of age and pregnant women based on the high prevalence of inadequate intakes of vitamin D and its important role in bone development and health and that vitamin D is of public health significance for infants 7 through 12 months of age based on its importance for growth and development during infancy.

We also proposed, at proposed § 101.9(c)(8)(ii), to require the declaration of potassium on foods for the general population. The AI for the general population is set at a level to maintain blood pressure, reduce the adverse effects of sodium chloride intake on blood pressure, and reduce the risk of recurrent kidney stones, but for infants, the AI is based on average potassium intake from breast milk and/or complementary foods (id.). Our analysis of NHANES 2003–2006 showed that 99 percent of infants ages 7 to 12 months have usual potassium intakes above the AI and that only 7 percent of children 1 through 3 years of age and 4 percent of pregnant women had usual potassium intakes above the AI from conventional foods or conventional foods plus dietary supplements, indicating that the adequacy of intakes is very low. We acknowledged, in the preamble to the proposed rule (79 FR 11879 at 11938) that, as a result of a FDAMA notification for a health claim about potassium, blood pressure, and stroke, foods may bear the following claim ''Diets containing foods that are good sources of potassium and low in sodium may reduce the risk of high blood pressure and stroke,'' on the label or labeling of any food product that meets the eligibility criteria described in the notification and meets the general requirements for a health claim (§ 101.14(e)(6)). This health claim pertains to the general population 2 years of age and older. Thus, because potassium is important in the risk reduction of these chronic diseases for children 2 years of age and older, we tentatively concluded that potassium is of public health significance to children 1 through 3 years of age, pregnant women, and lactating women and that, because of the benefits of adequate potassium intake in lowering blood pressure, data indicating low likelihood of potassium adequacy, and importance of establishing healthy dietary practices for later life, potassium is a nutrient of public health significance for infants 7 through 12 months of age, children 1 through 3 years of age, pregnant women, and lactating women. Thus, we proposed to require the labeling of vitamin D and potassium on foods represented or purported to be specifically for infants 7 through 12 months of age, children 1 through 3 years of age, or pregnant and lactating women based on the quantitative intake recommendations for vitamin D and potassium and the public health significance of these nutrients and did not provide for any exceptions for these subpopulations from the general requirement for declaration of vitamin D and potassium in proposed § 101.9(c)(8)(ii).

We did not receive comments regarding potassium and these subpopulations, so, other than replacing ''infants 7 to 12 months'' with ''infants through 12 months,'' we have finalized those provisions without change.

(Comment 452) One comment questioned the need for mandatory disclosure of vitamin D on the Nutrition Facts panel. The comment cited dietary intake data from food, beverages and supplements that suggests at least 75 percent of children ages 1 through 3 years have adequate intakes of vitamin D, not including sun exposure (Ref. 252). The comment said that mandatory declaration of vitamin D is not of value because relatively few foods have naturally occurring vitamin D, limitations on vitamin D addition to foods already exist, and vitamin D added to foods is already required on labeling. In addition, according to the comment, labeling can not necessarily help consumers achieve adequate intakes of vitamin D because it is not expected that all the required vitamin D will be provided by foods or supplements. Another comment noted that its products have many labels with very little label space and that using this label space for a declaration of 0 percent DV for vitamin D will limit its ability to provide other label information including information on other nutrients present in the products at significant levels.

(Response) We disagree with comments arguing against the mandatory declaration of vitamin D. We have determined that vitamin D is a nutrient of public health significance (79 FR 11879 at 11921 and 11938). The comment cited data that assessed usual intakes using the AI for vitamin D established in 1997 (Ref. 253). The IOM has since established an EAR for vitamin D (Ref. 38). Our analysis of NHANES data compared to the EAR showed 66 percent of children 1 through 3 years of age had inadequate intake of vitamin D from foods and supplements (79 FR 11879 at 11938).

We also disagree that mandatory declaration of vitamin D, including the declaration of zero percent DV, is not of value because few foods have naturally occurring vitamin D. As we discussed in the preamble to the proposed rule (79 FR 11879 at 11938) and part II.L, we identified vitamin D as a nutrient of public health significance for children 1 through 3 years of age based on the high prevalence of inadequate intakes of vitamin D and its important role in bone development and health (Ref. 198). Our analysis also shows that vitamin D intakes and status remain inadequate in the general population (79 FR 11879 at 11922). While limited label space may present challenges, the consideration for the mandatory declaration of vitamin D on the label is whether it will help consumers maintain healthy dietary practices.

While we acknowledge that some, but not all, vitamin D needs can be met by the body's exposure to sunlight, we determined the mandatory declaration of vitamin D based on the high prevalence of inadequate intakes of vitamin D and its important role in bone development and health (see part II.L). The mandatory declaration of vitamin D is intended to help consumers maintain healthy dietary practices and make healthy choices in context of a daily diet. The mandatory declaration of vitamin D also provides information to consumers about what foods are good sources of vitamin D and what foods do not contain vitamin D. Therefore, we have finalized this provision without change.

c. Voluntary declaration of vitamin A and vitamin C. We proposed to no longer require the declaration of vitamin A and vitamin C on foods for the general population. With respect to subpopulations, we noted, in the preamble to the proposed rule (79 FR 11879 at 11939) that our analysis of data from NHANES 2003–2006 showed that less than 2 percent of children 1 through 3 years of age had usual vitamin A intakes below the EAR from conventional foods or conventional foods plus dietary supplements and that, while 36 percent of pregnant women had usual intakes below the EAR from conventional foods and 22 percent had usual intakes below the

EAR for conventional foods plus dietary supplements, only 1 percent of these women had serum vitamin A levels that were considered to be indicative of a vitamin A deficiency. Furthermore, our analysis of data from NHANES 2003–2006 showed that neither vitamin A nor vitamin C is considered to have public health significance for children 1 through 3 years of age and pregnant women. Therefore, we tentatively concluded that vitamin A and vitamin C are not of public health significance among infants 7 through 12 months of age, children 1 through 3 years of age, and pregnant and lactating women, but we proposed to permit, but not require, the declaration of vitamin A and vitamin C on foods represented and purported to be specifically for infants 7 through 12 months, children 1 through 3 years of age, or pregnant and lactating women. As for other voluntary nutrients, the declaration of these nutrients would be required when these nutrients are added as nutrient supplements or claims are made about them (proposed § 101.9(c)(8)(ii)).

We did not receive comments regarding the voluntary declaration of vitamins A and C for subpopulations, so, other than replacing ''infants 7 to 12 months'' with ''infants through 12 months,'' we have finalized that provision without change.

d. Voluntary declaration of other vitamins and minerals. For the general population, we proposed to permit the voluntary declaration of vitamin E, vitamin K, vitamin B_6, vitamin B_{12}, thiamin, riboflavin, niacin, folate, biotin, pantothenic acid, phosphorus, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, chloride, and choline (proposed § 101.9(c)(8)(ii)). In the preamble to the proposed rule (79 FR 11879 at 11939), we said that vitamins and minerals other than iron, calcium, vitamin D and potassium for infants either have DRIs that are not based on chronic disease risk, heath-related conditions, or health-related physiological endpoints or are not shown to have public health significance due to the prevalence of a clinically relevant nutrient deficiency. For infants 7 to 12 months, children 1 through 3 years of age, and pregnant and lactating women, we tentatively concluded that the essential vitamins and minerals, other than iron, calcium, vitamin D and potassium, do not have public health significance and there is no basis for the declaration of these nutrients to be different from that proposed for the general population. Thus, proposed § 101.9(c)(8)(ii) would allow the voluntary declaration of vitamin E, vitamin K, vitamin B_6, vitamin B_{12}, thiamin, riboflavin, niacin, folate, biotin, pantothenic acid, phosphorus, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, chloride, and choline on foods represented or purported to be specifically for infants 7 to 12 months, children 1 through 3 years of age, pregnant women, or lactating women, under the requirements of this section, unless they are added to foods as a nutrient supplement or if the label or labeling makes a claim about them, in which case the nutrients would have to be declared.

We did not receive comments regarding the voluntary declaration of vitamin K, vitamin B_6, vitamin B_{12}, thiamin, riboflavin, niacin, folate, biotin, pantothenic acid, phosphorus, iodine, magnesium, zinc, copper, manganese, chromium, molybdenum, and chloride on foods represented or purported to be specifically for infants through 12 months of age, children 1 through 3 years of age, pregnant women, or lactating women. Therefore, other than replacing ''infants 7 to 12 months'' with ''infants through 12 months,'' we have finalized these provisions without change.

(Comment 453) One comment requested we reconsider mandatory declaration of vitamin E on nutrition labeling for children 1 through 3 years of age. The comment said that about 63 percent of children 12 to 24 months and 37 percent of children 24 to 48 months have vitamin E intakes below the EAR (Ref. 252). The comment also noted that encouraging an adequate intake of vitamin E in the diets of young children may encourage adequate consumption of foods with higher levels of vegetable fat.

(Response) We agree that vitamin E intakes are below the EAR and disagree that mandatory declaration of vitamin E is needed. Our analysis of NHANES data also has shown that intakes of children 1 through 3 years of age are below the EAR (79 FR 11879 at 11944). However, low intakes of vitamin E have not been associated with clinically relevant nutrient deficiency (Ref. 246). Therefore, consistent with the factors for mandatory or voluntary declaration of non-statutory nutrients (79 FR 11879 at 11889 and 11918, and part II.D), we have determined that vitamin E is not a nutrient public health significance for children 1 through 3 years of age and the general population.

The comment did not provide evidence to suggest that mandatory declaration of vitamin E may encourage adequate intake and consumption of foods with higher levels of vegetable fat, and we are not aware of any evidence to support that proposition. Therefore, we are not making changes in response to this comment.

(Comment 454) One comment supported the voluntary declaration of choline for pregnant and lactating women. The comment noted that choline has a role in preventing neural tube defects in infants and high intakes improve placental function and ease babies' response to stress during pregnancy. Another comment suggested that some nutrients should be considered for mandatory labeling, *e.g.,* choline and selenium as public health concerns. The comment also recommended that choline be considered for mandatory labeling on foods for pregnant and lactating women. The comment explained that mandatory labeling on foods in general, should be driven by the interest to reduce the risk of chronic diseases in adulthood, and should be revisited for foods for 7 months through 3 years to emphasize the role of nutrients in development.

(Response) We disagree that the declaration of choline and selenium should be mandatory. As the comment suggested, we have considered the relationship of nutrients and chronic disease risk, health-related conditions, or a health-related physiological endpoints (*i.e.* growth and development) in infants, children, and pregnant and lactating women to determine its mandatory or voluntary declaration on labeling. Based on our analysis of dietary intakes, we found no evidence of inadequate intakes of choline and selenium in these subpopulations. We also found no evidence for a substantial prevalence of chronic disease, health-related condition, or nutrient deficiency with clinical significance linked to choline and selenium in these subpopulations. Therefore, consistent with the factors for mandatory or voluntary declaration of these types of non-statutory nutrients (see part II.D), we have determined that choline and selenium are not nutrients of public health significance for infants through 12 months of age, children 1 through 3 years of age and pregnant and lactating women and have finalized the provision regarding voluntary declaration.

5. DRVs and RDIs for Infants Through 12 Months of Age

Our preexisting regulations do not include DRVs or RDIs for nutrients for infants, except for an RDI of protein of 14 grams. However, the proposed rule would establish a DRV or RDI for certain nutrients, and we explained, in the case

of polyunsaturated fat, monounsaturated fat, insoluble fiber, soluble fiber, added sugars, sugar alcohols, sodium, and fluoride, why we were not proposing to establish a DRV.

a. General comments.

(Comment 455) One comment recommended considering dietary intake data and public health need in addition to quantitative intake recommendations to determine appropriate RDIs for vitamins and minerals to be established for infants 7 months through 12 months of age and children 1 through 3 years of age. Another comment recommended that menu modeling and intake survey data should be a consideration in the establishment of certain DRVs as they provide insight on whether a DV is achievable, without compromising intake of another food group or nutrient and whether they align with dietary recommendations.

(Response) We agree dietary intake data and public health significance are important considerations in determining appropriate RDIs for vitamins and minerals. We consider public health significance in the context of developing RDIs for vitamins and minerals to refer to the existence of ''well-established'' scientific evidence from U.S. consensus reports that there is a relationship between a nutrient and chronic disease risk, a health-related condition, or a health-related physiological endpoint and where the intake of such nutrient is of general importance in the general U.S. population, *e.g.,* where intakes are generally too low or too high among the U.S. population. Thus, we established RDIs for vitamins and minerals based on the DRIs set by the IOM that reflect the most current science regarding nutrient requirements and associated disease risk, health-related condition, or health-related physiological endpoints (79 FR 11879 at 11926). While the DRI reports also consider dietary intake data, we also have analyzed more recent dietary intake data for these age groups (79 FR 11879 at 11944).

We acknowledge the comment suggesting that menu modeling and intake survey data could be a consideration in the establishment of certain DRVs. Dietary recommendations based on menu modeling may aim to achieve nutrient requirements, but are not the sole determining factor for establishing all DRVs. We agree that menu modeling can be considered in choosing a reference point for daily intake that is realistically achievable and practical in light of the current food supply and consumption patterns.

b. Calories. The preamble to the proposed rule (79 FR 11879 at 11939) stated that we have not established a reference calorie intake for infants. We noted that there is no quantitative intake recommendation for calories for infants and that we were not aware of scientific data and information on which we could rely to establish such a level (id.). Thus, we did not propose to establish a reference calorie intake level for infants 7 to 12 months.

We did not receive comments on this issue. Consequently, the final rule does not establish a reference calorie intake for infants though 12 months of age.

c. Total fat. Regarding total fat, the IOM set an AI of 30 grams/day for fat for infants 7 through 12 months of age based on the average intake of human milk and complementary foods. The AI provides a basis on which we can determine an appropriate DRV for total fat for infants 7 through 12 months, so we proposed to amend § 101.9(c)(9) to include a DRV of 30 grams for fat for infants 7 through 12 months of age.

We did not receive comments regarding the proposed DRV for infants, so the final rule establishes a DRV of 30 grams for fat for infants though 12 months of age.

d. Saturated fat, trans fat, cholesterol, dietary fiber, and sugars. Regarding saturated fat, *trans* fat, cholesterol, dietary fiber, and sugars, there are no quantitative intake recommendations from U.S. consensus reports available with respect to infants. Thus, we did not propose to establish DRVs for these nutrients for infants 7 through 12 months of age.

We did not receive comments on our decision not to establish DRVs for saturated fat, *trans* fat, cholesterol, and dietary fiber for infants. Thus, the final rule does not establish DRVs for infants though 12 months of age for these nutrients.

(Comment 456) One comment recommended establishing a DRV for sugars for infants and children and suggested that we work with the IOM to establish a DRV for sugar for this population.

(Response) We decline to establish a DRV for sugars for infants though 12 months of age and children 1 through 3 years of age. As discussed in part II.H.2, we are not aware of data or information related to a quantitative intake recommendation for sugars that we could use as the basis for a DRV for total sugars. The IOM reviewed the evidence on this topic in the Macronutrient report (IOM, 2002) and did not provide quantitative intake recommendations for infants and children.

e. Polyunsaturated fat, monounsaturated fat, insoluble fiber, soluble fiber, added sugars, and sugar alcohols. For polyunsaturated fat, monounsaturated fat, insoluble fiber, soluble fiber, added sugars, and sugar alcohols, there are no quantitative intake recommendations from U.S. consensus reports available with respect to infants. Thus, we did not propose to establish DRVs for these nutrients for infants 7 through 12 months of age.

We did not receive comments on our decision not to establish DRVs for polyunsaturated fat, monounsaturated fat, insoluble fiber, soluble fiber, added sugars, and sugar alcohols. Thus, the final rule does not establish DRVs for infants though 12 months of age for these nutrients.

f. Total carbohydrates. For total carbohydrates, the IOM set an AI of 95 grams/day for carbohydrates for infants 7 through 12 months of age based on the average intake of human milk and complementary foods; the AI provides a basis on which we can determine an appropriate DRV for total carbohydrate for this subpopulation that can assist consumers in maintaining healthy dietary practices among this subpopulation. Therefore, we proposed to amend § 101.9(c)(9) to establish a DRV of 95 grams for total carbohydrate for infants 7 through 12 months of age.

We did not receive comments regarding the proposed DRV of 95 grams for total carbohydrates for infants. Consequently, the final rule adopts the DRV of 95 grams for total carbohydrates for infants though 12 months of age.

g. Protein. For protein, the DV for protein for infants is an RDI, rather than a DRV. The preexisting RDI for infants is 14 grams/day for infants, but, in the preamble to the proposed rule (79 FR 11879 at 11940), we said we would revise the RDI to rely on current quantitative intake recommendations and that, in 2002, the IOM established an RDA for infants 7 through 12 months of 1.2 grams/kilogram/day based on nitrogen balance studies and using a reference body weight of 9 kilograms. The value 1.2 grams/kilogram/day × 9 kg equals 10.8 grams/day or a rounded value of 11 grams/day, yet we also noted that protein intakes are well above the current and proposed RDI. Mean protein intake for infants 6 to 11 months of age was 22 grams/day, well above the RDA of 11 grams/day. Thus, we proposed to revise § 101.9(c)(8)(iv) to establish an RDI of 11 grams for protein for infants 7 through 12 months of age.

We did not receive comments on our proposed RDI of 11 grams for infants, so the final rule, at § 101.9(c)(7)(iii) and (c)(8)(iv), establishes a RDI for protein of 11 grams for infants though 12 months of age.

h. Sodium. For sodium, we noted, in the preamble to the proposed rule (79 FR 11879 at 11940), that the IOM did not set a UL for sodium for infants 7 through 12 months of age due to insufficient data on adverse effects of chronic overconsumption in this age group. Thus, we did not propose a DRV for sodium for infants 7 through 12 months of age.

We did not receive comments regarding a DRV for sodium for infants. Thus, the final rule does not establish a DRV for sodium for infants though 12 months of age.

i. Fluoride. For fluoride, although the IOM set an AI for fluoride, the AI for infants 7 through 12 months is close to the EPA benchmarks for total fluoride intake. Additionally, we did not propose a DRV for fluoride for use in the labeling of foods for the general population because of a concern about excess intakes associated with dental fluorosis, and so, in the proposed rule, we tentatively concluded that a DRV for fluoride is not warranted for infants 7 through 12 months. Thus, we did not propose to establish a DRV for fluoride for infants 7 through 12 months of age.

We did not receive comments regarding establishment of DRVs for fluoride for infants. Thus, the final rule does not establish DRVs for fluoride for infants though 12 months of age.

j. Other vitamins and minerals. For vitamins and minerals, we reviewed current quantitative intake recommendations for vitamins and minerals for infants to determine appropriate RDIs for vitamins and minerals to be established in regulations for infants 7 through 12 months of age. In the preamble to the proposed rule (79 FR 11879 at 11940), we explained that we considered it important to establish RDIs for infants 7 through 12 months of age because infants in this age range transition from a diet of mostly breast milk and infant formula to infant cereal and baby foods, and labeling foods for this subpopulation with percent DV declarations can help parents make nutritious food choices. The DRIs (AIs and RDAs) provide a basis on which to determine RDIs for vitamins and minerals for this subpopulation. We considered it appropriate to use RDAs and, in the absence of RDAs, AIs to determine appropriate micronutrient RDIs for infants. We also stated that the IOM established DRIs based on scientific knowledge that update and supersede previous RDA recommendations. Consequently, we proposed to amend § 101.9(c)(8)(iv) to include a listing of RDIs for vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, vitamin B_{12}, folate, choline, riboflavin, niacin, vitamin B_6, calcium, iron, thiamin, biotin, pantothenic acid, phosphorous, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, chloride, and potassium for infants 7 through 12 months of age.

We did not receive comments regarding our proposed RDIs for vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, vitamin B_{12}, folate, choline, riboflavin, niacin, vitamin B_6, calcium, thiamin, biotin, pantothenic acid, phosphorous, iodine, magnesium, selenium, copper, manganese, chromium, molybdenum, chloride, and potassium for infants. Thus, the final rule adopts these RDIs for infants though 12 months of age without change.

(Comment 457) One comment would have us retain a DV for iron of 15 mg for infants given the importance of adequate iron in the diets of infants and young children and the prevalence of iron deficiency in children. The comment noted that published data reported 12 percent of infants aged 6 to 11 months have iron intakes from food, beverages, and supplements below the EAR (Butte 2010) and our analysis of NHANES data showed that 17.8 percent of infants aged 7 to 12 months have iron intakes from conventional foods only below the EAR.

(Response) We decline to revise the rule as suggested by the comment. We recognize the importance of adequate iron in the diets of infants. We acknowledge the dietary intake data and prevalence of iron deficiency for infants cited by the comment and point out that our analysis of NHANES data showed that 3 percent of infants aged 7 to 12 months have iron intakes below the EAR from food, beverages, and supplements. While we evaluated intakes, we consider that the DRI is the appropriate basis for establishing the DV for iron for infants because the DRI reports and its set of nutrient reference values are comprehensive reviews and applications of nutrition science research (79 FR 11879 at 11885).

(Comment 458) One comment questioned how a decrease in the DV for iron would affect iron fortification of foods for infants. The comment said that such a decrease in the DV could cause manufacturers to reduce iron fortification of products for this population group.

(Response) We disagree with the comment. The comment did not provide, and we are not aware of, any evidence to suggest that decreasing the DV for iron would impact iron fortification of foods for infants. DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements, not on potential changes in fortification of products. We recognize the importance of adequate iron intake in the diets of infants and intend to monitor the nutrient adequacy for this population and consider the need for consumer education.

(Comment 459) One comment asked that we use the current DV of 5 mg for zinc for infants as the DV for infants because previous RDA panels have recommended intakes of up to 10 mg for children 1 through 3 years of age and now recommend a RDA of 3 mg for infants and children 1 through 3 years of age. The comment also cited a study by Walravens et al. 1989 (Ref. 254) referenced by the IOM confirming the factorial approach and questioned the IOM's use of the Walraven baseline data minus 2 standard deviations to support for the EAR and suggested that reported dietary intake data, instead of standard deviations, maybe a more appropriate basis for EAR. The comment stated that lowering the DV to 3 mg/day may affect the availability and level of zinc fortification in foods and reduce intake levels without a full understanding of the potential impact in this sensitive population.

(Response) We decline to revise the rule as suggested by the comment. We are changing the DVs to reflect the most recent comprehensive reviews and applications of nutrition science research provided by current DRI reports and its set of nutrient reference values (see 79 FR 11879 at 11885). Modifying the reference value for zinc provided by these consensus reports is not warranted based on the scientific evidence to support the DRI.

We also disagree that using reported dietary intake data may be a more appropriate basis for the EAR infants. We note that the IOM established the EAR for zinc using a factorial approach and did not base the EAR on the growth data from the Walravens study (Ref. 226). We decline to comment on the IOM's rationale for the calculation used in confirming the factorial approach using the growth data cited by the Walraven study. We decline to speculate on how consumers may interpret % DV for zinc resulting from a recommended dietary pattern and whether they may inappropriately limit zinc intake. The comment did not provide, and we are not aware of, any evidence to suggest how consumers will react to the changes in percent DV as a result of changes to the DVs and whether they would inappropriately limit zinc intake. We recognize the importance of adequate zinc intake in

the diets of infants and intend to monitor the nutrient adequacy for this population and consider the need for consumer education.

We also have no evidence to suggest how that decreasing the DV for zinc would impact zinc fortification of foods for infants and decline to speculate on how availability and level of zinc fortification may change. DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements and not on potential changes in the fortification of products.

6. DRVs and RDIs for Children 1 Through 3 Years of Age

With respect to children 1 through 3 years of age, our preexisting regulations do not include DRVs or RDIs, except an RDI for protein of 16 grams for children less than 4 years of age. In the preamble to the proposed rule (79 FR 11879 at 11940 through 11941), we explained that we reviewed scientific evidence and current recommendations, as well as comments in response to the 2007 ANPRM to consider establishing DRVs and RDIs for nutrients for this subpopulation and to consider revisions to the current RDI for protein.

a. General comments.

(Comment 460) Several comments supported establishing DVs for children 1 through 3 years (13 through 48 months) that are consistent with the IOM's DRI recommendations for children 1 through 3 years age ranges.

In contrast, one comment suggested setting DVs specific for 4- to 8-year-old children because, according to the comment, setting a single DV that groups 4- to 8-year-old children with adults could lead to excessive intakes of some fortified vitamins and minerals and potentially increase the risk of adverse health effects from ingesting too much. The comment pointed out that the updated DVs for two nutrients, vitamin A and niacin, are the same as or higher than the IOM Tolerable Upper Intake Levels (ULs) for 4-to-8-year-olds.

Other comments suggested establishing RDIs and DRVs for children 4 to 13 years of age because product labeling based on RDIs for adults, in most cases, exceed the nutritional needs for children 4 to 13 years of age. The comments also noted that setting RDIs for children would provide an opportunity for more companies to formulate children's products to age-specific RDAs (rather than adult values which may not be appropriate for children's nutritional needs) and communicate the information to consumers via product labeling. One comment recommended that declarations of percent DV should be required for products targeted to children 4 through 13 years of age that contain nutrients for which this age-specific DRV or RDI is established.

(Response) We decline to revise the rule as suggested by the comments. While we recognize that nutritional needs of children aged 4 to 8 or 4 to 13 years are different from adults, we disagree with establishing RDIs for children aged 4 to 8 or 4 to 13 years due to concerns about excessive intake of nutrients above the UL or recommended intakes for these age groups. As noted in the preamble to the proposed rule (79 FR 11879 at 11928) and the accompanying memorandum to the file rule (Ref. 199), intakes of vitamins and minerals generally do not exceed the ULs under current RDIs that are based on a population coverage approach, except for zinc, vitamin A (preformed), iodine and folic acid among children 4 to 8 years old. In these few instances where total usual intakes of vitamins and minerals by children aged 4 to 8 years exceed corresponding ULs, we have determined that such intakes are not of public health significance.

With respect to the comment regarding niacin, the UL for niacin applies to niacin obtained from fortified foods and/or supplements and is based on flushing (burning, tingling sensation and reddening flush primarily on skin, arms and face) which is not considered a serious adverse effect. The UL for children was set by extrapolating downward from the UL for adults. While niacin intakes from fortified foods and dietary supplements may exceed the UL for children aged 4 to 8 years old (Refs. 194–195), no data were found to suggest that children have increased susceptibility to flushing effects from excess intake (Ref. 249).

We also disagree with establishing RDIs and DRVs for children 4 to 13 years of age and mandatory declaration of percent DV for products targeted to children 4 through 13 years of age to provide an opportunity for companies to formulate children's products to age-specific RDAs rather than adult values which may not be appropriate for children's nutritional needs. We recognize that RDAs for adults may be higher than the RDAs of children 4 through 8 years of age and 9 through 13 years of age. RDIs are intended to help persons to understand the relative significance of nutrients in the context of a total daily diet, to compare foods, and to plan general diets. They are not intended to be used to decide whether a particular individual's consumption of nutrients is appropriate. While RDIs are not precise values for certain age and sex groups, they function as an overall population reference to help consumers judge a food's usefulness in meeting overall daily nutrient requirements or recommended consumption levels and to compare nutrient contributions of different foods.

b. Calories. With respect to calories, we stated, in the preamble to the proposed rule (79 FR 11879 at 11940 through 11941), that several comments to the 2007 ANPRM supported establishing a DV for calories specifically for young children 1 through 3 years of age and that we considered it appropriate to establish a reference calorie intake level for children 1 through 3 years of age because we proposed to set DRVs using quantitative intake recommendations that are based on calories (*e.g.,* total fat, saturated fat, and dietary fiber). Because recommendations from the IOM, AHA, AAP, and the 2010 DGA for caloric intake range from 800 to 900 calories/day for children 1 year old, approximately 1,000 calories/day for children 2 years of age, and from 1,000 to 1,200 calories/day for children 3 years of age, we used an average of the range of these caloric intake recommendations (800 to 1,200 calories/day), *i.e.,* 1,000 calories/day, as a reasonable reference calorie intake level and proposed to amend § 101.9(c)(9) to provide a reference calorie intake level of 1,000 calories/day for children 1 through 3 years of age.

(Comment 461) One comment supported the reference calorie intake of 1,000 calories/day for children 1 through 3 years of age.

(Response) We agree with the reference calorie intake of 1,000 calories/day for labeling represented or purported to be for children 1 through 3 years of age. Thus, the final rule, at § 101.9(c)(9), establishes a reference calorie intake of 1,000 calories/day for children aged 1 through 3 years.

c. Total fat. In the preamble to the proposed rule (79 FR 11879 at 11941), we noted that there is no DRV for total fat for children ages 1 through 3 years, but a comment to the 2007 ANPRM recommended that 35 percent of the recommended 1,050 calories or 41 grams/day of fat be used to as the DRV for fat because it is the midpoint of the AAP/AHA recommendation and the IOM Acceptable Macronutrient Distribution Range (AMDR) for 1 through 3 year olds. We agreed that 35 percent of calories from fat for children 1 through 3 years of age serves as an appropriate basis on which to set the DRV for total fat and would be consistent with AHA and AAP recommendations that 30 to 40 percent

of calories consumed by children 12 to 24 months of age and 30 to 35 percent of calories consumed by children 24 through 48 months of age should come from fat. Therefore, we tentatively concluded that 35 percent of total calories from fat (*i.e.,* 39 grams using the proposed reference calorie intake level of 1,000 calories/day) is an appropriate DRV for total fat for children 1 through 3 years of age, and we proposed to amend § 101.9(c)(9) to establish a DRV of 39 grams for fat for children 1 through 3 years of age.

(Comment 462) One comment would increase the DRV for total fat for children 1 through 3 years of age to 41 grams, given the importance of an adequate intake of total fat in this population for healthy development and growth. The comment noted that this level of total fat would be 37 percent of total calories from fat (based on 1,000 calories/day reference calorie intake level) which is within the AMDR of 30 to 40 percent total calories from fat. The comment cited dietary intake data suggesting that 23 percent (12 to 23 months) and 47 percent (24 to 48 months) of children are below the AMDR. The comment noted that it is important for the total fat DV to help encourage adequate fat intake.

(Response) We decline to increase the DRV for total fat. In the preamble to the proposed rule (79 FR 11879 at 11941), we determined that 35 percent of calories from fat, based on a 1,000 calorie/day reference calorie intake level, is an appropriate basis for the DRV for total fat because it aligns with the AHA and AAP recommendations that 30 to 40 percent of calories consumed by children 12 through 24 months of age and 30 to 35 percent of calories consumed by children 24 through 48 months of age should come from fat and is consistent with our proposed approach to setting the DRV for total fat for the general population (Ref. 255). We acknowledge the dietary intake data suggesting the total fat intake of children is below the AMDR. This calculation yields a DRV of 39 grams.

We disagree that the purpose of the total fat DV is to encourage fat intake. The DVs are intended to help persons to understand the relative significance of nutrients in the context of a total daily diet, to compare foods, and to plan general diets. They are not intended to be used to decide whether a particular individual's consumption of nutrients is appropriate.

Thus, the final rule, at § 101.9(c)(9), establishes a DRV of 39 grams for total fat for children aged 1 through 3 years.

d. Saturated fat, trans fat, and cholesterol. For saturated fat, *trans* fat, and cholesterol, we stated, in the preamble to the proposed rule (79 FR 11879 at 11941), that there are no DRVs for children 1 through 3 years of age. Based on the scientific evidence in the 2010 DGA to support that Americans 2 years of age and older consume less than 10 percent of calories from saturated fat and less than 300 mg/day of cholesterol, we tentatively concluded that it would be appropriate to set a DRV of 10 grams for saturated fat, based on 10 percent of total calories from saturated fat and using the proposed reference calorie intake level of 1,000 calories/day, which equals 11 grams, rounded down to 10 grams, and a DRV of 300 mg for cholesterol for children 1 through 3 years of age. We proposed to amend § 101.9(c)(9) to establish a DRV of 10 grams for saturated fat and a DRV of 300 mg for cholesterol for children 1 through 3 years of age. We declined to propose a DRV for *trans* fat because the scientific evidence from the IOM and the 2010 DGA did not provide any specific appropriate levels of intake.

(Comment 463) One comment recommended using the DRV of 12 grams for saturated fat for children 1 through 3 years of age. The comment noted that this value represents 10.7 percent of calories from saturated fat based on a 1,000 calorie diet and is consistent with the diets of about 25 percent of children between 12 and 47 months, an indication that this level of intake is achievable.

(Response) We decline to change the DRV for saturated fat as suggested by the comment. In establishing the DRV for saturated fat, we considered that cardiovascular disease can begin in childhood and the scientific evidence in the 2010 DGA that support Americans 2 years of age and older consuming less than 10 percent of calories from saturated fat (79 FR 11879 at 11941). We disagree that the DRV for saturated fat should be based on dietary intake data that suggest that a level of 12 grams is achievable. DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements, not on levels of intakes that are achievable. Thus, the final rule, at § 101.9(c)(9), establishes a DRV of 10 grams for saturated fat for children aged 1 through 3 years. Additionally, on our own initiative, we have replaced ''saturated fatty acids'' in the table with ''saturated fat'' for consistency in how we refer to saturated fat. We also have replaced ''Unit of measurement'' with ''Unit of measure'' in the table for consistency with the introductory sentence to § 101.9(c)(9).

We did not receive comments regarding our tentative decision not to establish a DRV for *trans* fat or the proposed DRV of 300 mg for cholesterol for children aged 1 through 3 years. Thus, the final rule establishes a DRV of 300 mg for cholesterol for children aged 1 through 3 years and does not establish a DRV for *trans* fat.

e. Polyunsaturated fat, monounsaturated fat, sugars, insoluble fiber, soluble fiber, added sugars, and sugar alcohols. For polyunsaturated fat, monounsaturated fat, sugars, added sugars, insoluble fiber, soluble fiber, and sugar alcohols, we stated, in the preamble to the proposed rule (79 FR 11879 at 11941), that there are no DRVs for children 1 through 3 years of age. We recognized the essential nature of α-linolenic acid in the diet, but we said that, for children 1 through 3 years of age, DRIs or other data and information were not available on which we could rely to establish DRVs for polyunsaturated fat, monounsaturated fat, sugars, added sugars, insoluble fiber, soluble fiber, and sugar alcohols (id.). Therefore, we tentatively concluded that there was no basis for setting DRVs for these nutrients and did not propose DRVs for polyunsaturated fat, including n-3 or n-6 polyunsaturated fatty acids, monounsaturated fat, sugars, added sugars, soluble fiber, insoluble fiber, or sugar alcohols for children 1 through 3 years of age.

We did not receive comments on our tentative decision not to establish DRVs for polyunsaturated fat, monounsaturated fat, sugars, insoluble fiber, soluble fiber, and sugar alcohols. Thus, the final rule does not establish DRVs for children 1 through 3 years of age for these nutrients.

(Comment 464) Some comments agreed with not defining DVs for added sugars. One comment recommended establishing a DRV for added sugar for children.

(Response) We received many comments on defining a DRV for added sugars and explain, in part II.H.3.o, that we are establishing a DRV for added sugars for children and adults 4 years of age and older of no more than 10 percent of total calories, or 50 grams using a 2,000 calorie intake reference amount based on food pattern modeling. For the reasons discussed in part II.H.3.o, we are also establishing a DRV of 25 grams of added sugars for children 1 through 3 years of age based on food pattern modeling. Using the 1,000 calorie intake reference amount for children 1 through 3 years of age and the DRV of no more than 10 percent of total calories, the DRV for children 1 through 3 years of age is 25 grams (1,000

calories × 0.1 = 100 calories and 100 calories ÷ 4 calories per gram for carbohydrates = 25 grams). Thus, the final rule, at § 101.9(c)(9), establishes a DRV of 25 grams for added sugars for children ages 1 through 3 years of age.

f. Total carbohydrates. In the preamble to the proposed rule (79 FR 11879 at 11941), we said that, for total carbohydrates, there is not a DRV for children 1 through 3 years of age. We noted, however, that we were proposing a DRV for total carbohydrate for the general population based on the percentage of calories in a 2,000 calorie diet remaining after the sum of the DRV for fat (30 percent) plus the DRV for protein (10 percent) have been subtracted and that we considered this method to be appropriate for setting a DRV for total carbohydrate for children 1 through 3 years of age (id.). We also stated that total calories (100 percent) minus the proposed DRV for total fat (35 percent of calories) and the proposed DRV for protein (5 percent of calories) equals 60 percent of calories from total carbohydrate. A value of 60 percent of total calories from total carbohydrates also falls within the IOM AMDR recommendation of 45 to 65 percent of calories from carbohydrates for children 1 through 3 years of age. Therefore, we tentatively concluded that an appropriate DRV for total carbohydrate is 60 percent of calories (*i.e.,* 150 grams using the proposed reference calorie intake level of 1,000 calories/day), and we proposed to amend § 101.9(c)(9) to set a DRV of 150 grams for total carbohydrate for children 1 through 3 years of age.

We did not receive comments regarding the proposed DRV of 150 grams for children 1 through 3 years of age, so the final rule adopts this DRV without change.

g. Dietary fiber. In the preamble to the proposed rule (79 FR 11879 at 11941), we stated that there is not a DRV for dietary fiber for children 1 through 3 years of age, but we agreed with a comment to an ANPRM that an AI of 14 grams/1,000 calories for dietary fiber for children 1 through 3 years of age should be used to set a DRV for dietary fiber to be consistent with how other proposed DRVs are being set. Additionally, because we proposed a reference calorie intake level of 1,000 calories/d for this subpopulation, we proposed to amend § 101.9(c)(9) to establish a DRV of 14 grams for dietary fiber for children 1 through 3 years of age.

We did not receive comments regarding the proposed DRV of 14 grams for fiber for children 1 through 3 years of age. Thus, the final rule adopts this DRV without change.

h. Protein. Under our preexisting regulations, at § 101.9(c)(7)(iii), the RDI for protein for children younger than 4 years of age was based on the 1989 RDA for protein of 16 grams/day. Taking into account current recommendations and protein intakes, we noted, in the preamble to the proposed rule (79 FR 11879 at 11942), that protein intakes are well above the current RDI, with the mean protein intake for children 12 to 23 months of age being 44 grams/day, well above the RDA of 13 grams/day, and the midpoint of the AMDR of 5 to 20 percent calories from protein (*i.e.,* 12.5 percent of calories from protein or 31 grams/day). The protein AMDR for children 1 through 3 years of age is 5 to 20 percent of calories, and the RDA is approximately 5 percent of calories. Given the proposed reference calorie intake level and the approaches used for the proposed DRVs for fat and carbohydrate that are based on percent of calories, we tentatively concluded that, as with the general population, the DV for protein for children 1 through 3 years of age should be a DRV, rather than an RDI (using the RDA) and that a DRV for protein should be based on 5 percent of 1,000 calories or 50 calories which equals 12.5 grams or, when rounded up, 13 grams. We proposed to amend § 101.9(c)(7)(iii) and (c)(9) to establish a DRV for protein of 13 grams for children 1 through 3 years of age.

(Comment 465) One comment recommended retaining the current DV of 16 grams for protein or using 10 percent of calories from protein. The comment noted that children 24 to 47 months have 13 to 19 percent of energy intakes from protein, respectively. The comment said that the proposed DV of 13 grams appears to be low relative to the protein that would be expected to be contributed from a diet that supplies the appropriate servings of foods from the recommended food groups, including milk, meat/poultry and beans and other legumes.

(Response) We decline to retain a DV of 16 grams for protein. In the preamble to the proposed rule (79 FR 11879 at 11942), we discussed a comment to the 2007 ANPRM recommending the DV for protein be maintained at 16 grams. We declined to keep the DV for protein at 16 grams, in part, because protein intakes are well above the current RDI. Mean protein intake for children 12 to 23 months of age was 44 grams/day, well above the RDA of 13 grams/day and the midpoint of the AMDR of 5 to 20 percent calories from protein (*i.e.,* 12.5 percent of calories from protein or 31 grams/day, which we rounded up to 13 grams). The protein AMDR for children 1 through 3 years of age is 5 to 20 percent of calories and the RDA is approximately 5 percent of calories. Thus, a DRV for protein should be based on 5 percent of 1,000 calories or 50 calories which equals 12.5 grams or, when rounded up, 13 grams, and the final rule, at § 101.9(c)(7)(iii) and (c)(9), establishes a DRV for protein of 13 grams for children 1 through 3 years of age.

i. Sodium. In the preamble to the proposed rule (79 FR 11879 at 11942), we noted that, for the general population, we proposed to establish a DRV based on the UL for sodium and that there is no DRV for sodium for children 1 through 3 years of age. We also noted that the IOM derived the UL for children 1 through 3 years of age by extrapolation from the adult UL of 2,300 mg/day based on observational studies showing that blood pressure increases with age into adulthood and the recognition that risk factors for CVD, such as high blood pressure and atherosclerosis, occur in childhood (id.). We proposed to amend § 101.9(c)(9) to establish a DRV of 1,500 mg for sodium for children 1 through 3 years of age.

We did not receive comments regarding the DRV of 1500 g for sodium for children 1 through 3 years of age. Thus, the final rule, at § 101.9(c)(9), establishes a DRV of 1,500 mg for sodium for children 1 through 3 years of age.

j. Fluoride. There is not a DV for fluoride for children 1 through 3 years of age. In the preamble to the proposed rule (79 FR 11879 at 11942), we said that, although the IOM recognized fluoride as a trace mineral that is important for public health by setting an AI based on evidence of its role in reducing the risk of dental caries, we tentatively concluded that a DRV should not be established for fluoride. The proposed rule did not contain a DRV for fluoride for children 1 through 3 years of age.

We did not receive comments regarding the establishment of DRVs for fluoride for children 1 through 3 years of age. Thus, the final rule does not establish a DRV for fluoride for children 1 through 3 years of age.

k. Other vitamins and minerals. In the preamble to the proposed rule (79 FR 11879 at 11942 through 11943), we stated that the IOM's quantitative intake recommendations (AIs and RDAs) provide a basis on which to determine RDIs for vitamins and minerals for children 1 through 3 years of age. We explained that the RDA, when available, is the best estimate of an intake level that will meet the nutrient goals of practically all consumers who would use the Nutrition Facts label and that,

while AIs have less certainty than RDAs, AIs represent goals for nutrient intake for individuals and provide the best estimate based on current science for use in setting RDIs for such nutrients (see id.). Therefore, using the RDAs and AIs, we proposed to amend § 101.9(c)(8)(iv) to establish RDIs for vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, vitamin B_{12}, folate, choline, riboflavin, niacin, vitamin B_6, calcium, iron, thiamin, biotin, pantothenic acid, phosphorous, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, chloride, and potassium for children 1 through 3 years of age.

We did not receive comments regarding our proposed RDIs for vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, vitamin B_{12}, folate, choline, riboflavin, niacin, vitamin B_6, calcium, thiamin, biotin, pantothenic acid, phosphorous, iodine, magnesium, selenium, copper, manganese, chromium, molybdenum, and chloride for children 1 through 3 years of age. Thus the final rule adopts these RDIs for children 1 through 3 years of age without change.

(Comment 466) One comment said that a DV for potassium of 3,000 mg for children aged 1 through 3 years is unrealistic and may promote an unbalanced diet. The comment said that the DV for potassium should be calculated using a 1,000 calorie diet instead of the 1,372 calorie factor used by the IOM for 1 through 3 year olds. The comment requested a DV of 2,300 mg given the reference caloric intake of 1,000 for children ages 1 through 3 years.

Another comment expressed concern that, with a DV of 3,000 mg, several foods products would no longer be considered a ''good source'' of potassium.

(Response) We decline to establish a DV of 2,300 mg for potassium, and we disagree with the comment regarding foods that would no longer be considered as a ''good source'' of potassium. In the preamble to the proposed rule (79 FR 11879 at 11942), we discussed how we had considered comments to the 2007 ANPRM suggesting that we use 1,800 or 2,000 mg/day potassium as the basis for the RDI for potassium; we said that it would be inconsistent with the approach for the general population. Selecting a number other than a RDA or AI, when there is one, is inconsistent with our approach for establishing DVs. We rely on the DRI reports and its set of nutrient reference values for establishing the DVs because they are comprehensive reviews and applications of nutrition science research. We acknowledge that current potassium intakes are below the proposed DV of 3,000 mg. However, we disagree that the DV for potassium may promote an unbalanced diet. Dietary sources of potassium are found in all food groups, notably in vegetables and fruits, and milk and milk products (Ref. 30). Promoting the development of healthy eating patterns that will be associated with adequate potassium intake later in life is important because chronic conditions such as elevated blood pressure, bone demineralization, and kidney stones likely result from inadequate potassium intakes over an extended period of time, including childhood (Ref. 256).

We disagree that DVs should be set based on realistic intakes or eligibility to make a nutrient content claim. The DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements, not on levels of intakes that are achievable or eligibility to make nutrient content claims.

(Comment 467) One comment would have us retain a DV for iron of 10 mg of children 1 through 3 years given the importance of adequate iron in the diets of infants and young children and the prevalence of iron deficiency in children. The comment noted that dietary intake data in children aged 12 to 24 months suggests that children may be consuming less heme iron than assumed in the determination of the IOM EAR so the EAR may be too low to achieve the requirement of absorbed iron. However, the comment did not provide an amount or percentage of heme iron being consumed from current intakes and also cited data from published and unpublished sources.

(Response) We decline to revise the rule as suggested by the comment. We recognize the importance of adequate iron in the diets of infants and young children. As for the statement that children may be consuming less heme iron than assumed in the IOM's determination of the EAR, as the comment provided data from one published study reflecting dietary intake data from 2002 and did not provide estimates of the heme iron consumed or total iron absorbed, we cannot determine from the information provided by the comment that the EAR may be too low to achieve the requirement of absorbed iron.

Furthermore, selecting a number other than a RDA or AI is inconsistent with our approach for establishing DVs. We rely on the DRI reports and its set of nutrient reference values for establishing the DVs because they are comprehensive reviews and applications of nutrition science research (79 FR 11879 at 11885).

(Comment 468) One comment questioned how a decrease in the DV for iron would affect iron fortification of foods for toddlers. The comment said that such a decrease in the DV could cause manufacturers to reduce iron fortification of products for this population group.

(Response) We disagree with the comment. The comment did not provide, and we are not aware of, any evidence to suggest that decreasing the DV for iron would impact iron fortification of foods for toddlers. DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements, not on potential changes in fortification of products. We recognize the importance of adequate iron intake in the diets of young children and intend to monitor the nutrient adequacy for this population and consider the need for consumer education.

(Comment 469) One comment asked that we use the current DV of 5 mg for zinc for infants as the DV for children 1 through 3 years of age because previous RDA panels have recommended intakes of up to 10 mg for children 1 through 3 years of age and now recommend a RDA of 3 mg for infants and children 1 through 3 years of age. The comment also cited a study by Walravens et al. 1989 (Ref. 254) referenced by the IOM confirming the factorial approach and questioned the IOM's use of the Walravens baseline data minus 2 standard deviations to support for the EAR and suggested that reported dietary intake data, instead of standard deviations, maybe a more appropriate basis for EAR. The comment said that the zinc consumption from a recommended dietary pattern for children 1 through 3 years of age would be at least 6 mg, or 200 percent of the proposed DV and that consumers would likely be confused by these high amounts per serving and could take steps to inappropriately limit zinc intake. The comment stated that lowering the DV to 3 mg/day may affect the availability and level of zinc fortification in foods and reduce intake levels without a full understanding of the potential impact in this sensitive population.

(Response) We decline to revise the rule as suggested by the comment. We are changing the DVs to reflect the most recent comprehensive reviews and applications of nutrition science research provided by current DRI reports and its set of nutrient reference values (see 79 FR 11879 at 11885).

We also disagree that using reported dietary intake data may be a more appropriate basis for the EAR children 1 through 3 years of age. We note that the IOM established the EAR for zinc using a factorial approach and did not base the EAR on the growth data from the Walravens study (Ref. 226).

The comment did not provide, and we are not aware of, any evidence to suggest how consumers will react to the changes in percent DV as a result of changes to the DVs and whether they would inappropriately limit zinc intake. We recognize the importance of adequate zinc intake in the diets of young children and intend to monitor the nutrient adequacy for this population and consider the need for consumer education.

We also have no evidence to suggest how that decreasing the DV for zinc would impact zinc fortification of foods for toddlers and decline to speculate on how availability and level of zinc fortification may change. DVs are established based on DRIs set by the IOM that reflect the most current science regarding nutrient requirements and not on potential changes in the fortification of products.

7. DRVs and RDIs for Pregnant Women and Lactating Women

The proposed rule would establish certain DRVs and RDIs for pregnant women and lactating women.

a. Calories. The proposed rule would use the 2,000 reference calorie intake level for setting DRVs for pregnant women and lactating women (§ 101.9(c)(9)). In the preamble to the proposed rule (79 FR 11879 at 11943), we explained that the calorie needs for pregnant women and lactating women are similar to the general population, and few products are purported for pregnant and lactating women. Thus, because the reference calorie intake for the general population is 2,000, we proposed to use the 2,000 reference calorie intake level for setting DRVs for pregnant women and lactating women (§ 101.9(c)(9)).

We did not receive comments on our proposed 2,000 reference calorie intake level for setting DRVs for pregnant women and lactating women. Thus, we have finalized the provision without change on this point. However, on our own initiative, we have made a grammatical change to the rule's mention of ''pregnant and lactating women'' to refer, instead, to ''pregnant women and lactating women.'' We have made this change to clarify that the rule is referring to two groups (pregnant women and lactating women) instead of one group.

b. Total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber. For total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber, we explained, in the preamble to the proposed rule (79 FR 11879 at 11943), that the quantitative intake recommendations for total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber for pregnant and lactating women are generally similar to the general population. Thus, we tentatively concluded that the DRVs for total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber for pregnant and lactating women should remain the same as for the general population, and so we proposed to amend § 101.9(c)(9) to establish DRVs for pregnant and lactating women using the proposed DRVs for the general population for total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber.

We did not receive comments on our proposal to establish DRVs for total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber for pregnant and lactating women based on the DRVs for the general population for total fat, saturated fat, cholesterol, total carbohydrate, sodium, and dietary fiber. Thus, we have finalized these provisions without change.

c. Trans fat, polyunsaturated fat, monounsaturated fat, insoluble fiber, soluble fiber, sugars, added sugars, and sugar alcohols. For *trans* fat, polyunsaturated fat, monounsaturated fat, soluble fiber, insoluble fiber, sugars, added sugars, and sugar alcohols, in the preamble to the proposed rule (79 FR 11879 at 11943), we said that we did not propose DRVs for these nutrients for the general population because of a lack of quantitative intake recommendations. Because quantitative intake recommendations are lacking for these nutrients for pregnant and lactating women, we did not propose to establish DRVs for *trans* fat, polyunsaturated and monounsaturated fat, soluble fiber, insoluble fiber, sugars, added sugars, or sugar alcohols for pregnant and lactating women.

We did not receive comments on our proposal not to establish DRVs for *trans* fat, polyunsaturated and monounsaturated fat, insoluble fiber, soluble fiber, sugars, or sugar alcohols for pregnant and lactating women. Thus, the final rule does not establish DRVs for *trans* fat, polyunsaturated and monounsaturated fat, insoluble fiber, soluble fiber, sugars, or sugar alcohols for pregnant and lactating women.

However, with respect to added sugars, we received many comments on defining a DRV for added sugars for children and adults 4 years of age and older and explain, in part II.H.3.o, that we are establishing a DRV for added sugars for children and adults 4 years of age and older of no more than 10 percent of total calories, or 50 grams using a 2,000 calorie intake reference amount based on food pattern modeling. For the reasons discussed in part II.H.3.o, we also are establishing a DRV for added sugars for pregnant women and lactating women of no more than 10 percent of total calories, or 50 grams using a 2,000 calorie intake reference amount based on food pattern modeling. Thus, the final rule at § 101.9(c)(9), establishes a DRV of 50 grams for added sugars for pregnant women and lactating women.

d. Protein. Our preexisting regulations, at § 101.9(c)(7)(iii), establish RDIs of 60 grams of protein for pregnant women and 65 grams of protein for lactating women based on the highest 1989 RDAs for pregnant and lactating women. In the preamble to the proposed rule (79 FR 11879 at 11943), we noted that the IOM established 71 grams/day protein as the RDA for pregnant and lactating women based on the needs for maternal and fetal development and human milk production. Because the RDA for protein during both pregnancy and lactation is the same, and given that most foods represented or purported to be specifically for pregnant women are also represented or purported to be specifically for lactating women, we tentatively concluded that it would be appropriate to establish a single RDI of 71 grams applicable to both pregnant and lactating women and that the DV for protein for pregnant and lactating women should remain an RDI (using the RDA) instead of a DRV because the DRV approach used to calculate protein for the general population based on 10 percent of 2,000 calories, which equals 50 grams of protein/day, falls short of the recommended protein needs of pregnant and lactating women of 71 grams/day. Thus, we proposed to amend § 101.9(c)(7)(iii) to establish an RDI of 71 grams for protein for pregnant and lactating women.

We did not receive comments on the proposed RDI of 71 grams for protein for pregnant and lactating women. Thus, we have finalized this provision without change.

e. Fluoride. For fluoride, we did not propose to establish a DRV for pregnant or lactating women because we were not proposing a DRV for fluoride in the general population.

We did not receive comments regarding the establishment of a DRV for fluoride for pregnant and lactating

women. Thus, the final rule does not establish a DRV for fluoride for pregnant and lactating women.

f. Vitamins and minerals. For vitamins and minerals, in the preamble to the proposed rule (79 FR 11879 at 11943), we considered it appropriate to establish RDIs for pregnant and lactating women for vitamins and minerals that have DRIs, using population-coverage RDAs and AIs, instead of population-weighted EARs. We proposed to establish a single set of RDIs intended for both pregnant women and lactating women because nutrient needs during pregnancy and lactation are similar. Thus, we proposed to amend § 101.9(c)(8)(iv) to establish RDIs as set forth previously for vitamin A, vitamin C, vitamin D, vitamin E, vitamin K, vitamin B_{12}, folate, choline, riboflavin, niacin, vitamin B_6, calcium, iron, thiamin, biotin, pantothenic acid, phosphorous, iodine, magnesium, zinc, selenium, copper, manganese, chromium, molybdenum, chloride, and potassium for pregnant and lactating women.

We did not receive comments with respect to these DRVs and RDIs for pregnant and lactating women, and so we have finalized these provisions without change.

P. Dietary Supplements

Our preexisting regulations specific to dietary supplement nutrition labeling appear in § 101.36. Many requirements in § 101.36 are consistent with the requirements for the nutrition labeling of conventional foods in § 101.9, and there are references throughout § 101.36 to requirements established in § 101.9.

The proposed rule would amend both the content and format of the Supplement Facts label to correspond to the Nutrition Facts label.

1. Mandatory Dietary Ingredients

Our preexisting regulations, at § 101.36(b)(2), provide information on dietary ingredients that have an RDI or a DRV as established in § 101.9(c)(8)(ii) and (c)(9). These dietary ingredients are known as the ''(b)(2)-dietary ingredients.'' Of these 15 nutrients, vitamin A, vitamin C, calcium, and iron must be listed in the Supplement Facts label for a dietary supplement when the quantitative amount by weight exceeds the amount that can be declared as zero in the nutrition labeling of foods in accordance with § 101.9(c). Section 101.36(b)(2) states that any (b)(2)-dietary ingredients that are not present, or that are present in amounts that can be declared as zero in § 101.9(c), must not be declared (*e.g.,* amounts corresponding to less than 2 percent of the RDI for vitamins and minerals). The regulation also requires, in § 101.36(b)(2), that calories from saturated fat and polyunsaturated fat, monounsaturated fat, soluble fiber, insoluble fiber, sugar alcohol, other carbohydrate, and § 101.9(c)(8)(iv) or (c)(9) vitamins and minerals other than vitamin A, vitamin C, calcium, and iron may be declared, but they must be declared when they are added to the product for purposes of supplementation, or when a claim is made about them.

We proposed to update the list of (b)(2)-dietary ingredients to maintain consistency with the proposed requirements for nutrition labeling of foods in § 101.9. Therefore, proposed § 101.36(b)(2)(i) would: (1) No longer require declaration of vitamin A, vitamin C, or Calories from fat; (2) require vitamin D and potassium; (3) require the declaration of added sugars; and (4) retain the other (b)(2)-dietary ingredients as mandatory declarations. We also proposed to amend § 101.36(b)(2)(i), (b)(2)(i)(B)(1), and (b)(2)(iii)(G) to remove the requirement for declaration of ''Calories from fat.''

We did not receive comments on these proposed changes to the Supplement Facts label, and so, with the exception of replacing ''sugars'' with ''total sugars'' in § 101.36(b)(2)(i), we have finalized the provisions without change.

We note that we did receive comments, in general, on removing the declaration of vitamins A and C and on requiring the declaration of vitamin D and potassium; we discuss those comments in part II.L.2 and II.L.3. We also received comments on removing the requirement for declaration of ''Calories from fat;'' we discuss those comments in part II.E.1.

2. Folate and Folic Acid

The preamble to the proposed rule (79 FR 11879 at 11947) explained that folate is a nutrient found in conventional foods, whereas folic acid is the synthetic form of folate that is added to fortified conventional foods and dietary supplements. Because of the difference in bioavailability between naturally occurring folate and synthetic folic acid, we proposed to:

- Amend § 101.9(c)(8)(v) such that the term ''folate'' would be used in the labeling of conventional foods that contain either folate alone or a mixture of folate and folic acid;
- amend § 101.36(b)(2)(i)(B) and (b)(2)(i)(B)(2) to specify that ''folic acid'' is the term used to declare folic acid content of dietary supplements; and
- remove ''folate'' and ''folacin'' from the list of synonyms that may be used to declare folic acid on the Supplement Facts label.

(Comment 470) Many comments opposed allowing only the use of the term ''folic acid'' on dietary supplements. The comments said that dietary supplements can contain folate.

(Response) As discussed in part II.N.3.b, the final rule requires that the Supplement Facts label declare folate in mcg DFE, a percent DV based on mcg DFE, and that the mcg of folic acid be stated in parenthesis when folic acid is added as a nutrient supplement to a dietary supplement. In doing so, there will be consistency with the use of the term folate in labeling of both conventional foods and dietary supplements. In addition, the mcg DFE reflects the fact that folic acid is more bioavailable than folate and is the basis of the DV. By requiring the declaration of the mcg DFE folate, a percent DV based on mcg DFE, and the mcg of folic acid in parentheses on dietary supplements when folic acid is added as a nutrient supplement, consumers will be aware of the type and amount of folate or folic acid in the dietary supplement.

The final rule also removes ''folacin'' from the list of synonyms that may be used for folate in the Nutrition Facts label in § 101.9(c)(8)(v) and the Supplement Facts label in § 101.36(b)(2)(i)(B)(2)). In addition, the final rule removes the term ''folic acid'' from the list of synonyms that may be added in parentheses immediately following ''folate'' on the Nutrition Facts label in § 101.9(c)(8)(v) or in place of the term ''folate'' on the Supplement Facts label in § 101.36(b)(2)(B)(2) because we are now requiring that the terms ''folate'' and ''folic acid'' be included, when declared, on both the Nutrition and Supplement Facts label.

3. Units of Measure

The proposed rule would amend § 101.9(c)(8)(iv) to replace ''IU'' for the RDIs for vitamin A, vitamin D, and vitamin E with mcg RAE for vitamin A, mcg for vitamin D, and mg α-tocopherol for vitamin E. The proposed rule would quantify and declare folate and folic acid in ''mcg DFE'' instead of ''mcg.'' For consistency in nutrition labeling of foods and dietary supplements, the proposed rule also would amend § 101.36(b)(2)(i)(B)(3) to require that, when β-carotene is included in parentheses following the percent statement for vitamin A, it should be declared using ''mcg'' (representing mcg RAE) as the unit of measure. In addition, under § 101.36(b)(2)(ii)(B), the

proposed units of measure for vitamin D, vitamin E, and folate in § 101.9(c)(8)(iv) would be used in the declaration of vitamin D, vitamin E, and folic acid in the Supplement Facts label.

(Comment 471) Some comments disagreed with our proposal to replace "IU" for the RDIs for vitamin A, vitamin D, vitamin E with mcg RAE for vitamin A, mcg for vitamin D, and mg α-tocopherol for vitamin E.

(Response) We address these comments in part II.N.4. The final rule, at § 101.9(c)(8)(iv), revises the units of measure to be mcg RAE for vitamin A, mcg for vitamin D (with the allowance of voluntary declaration of IUs), and mg α-tocopherol for vitamin E, and § 101.36(b)(2)(ii)(B), therefore, adopts the same units of measure for vitamin D, vitamin E, and folate.

Additionally, we did not receive comments on the proposed changes to the declaration of β-carotene at § 101.36(b)(2)(i)(B)(3), so we have finalized that provision without change.

(Comment 472) One comment said we should adopt a unit of measure for fluoride of mg per liter (mg/L) rather than mg/servings.

(Response) We address this comment in part II.K.3. The final rule does not adopt mg/L as the unit of measure for fluoride.

(Comment 473) The proposed rule, at § 101.36(b)(2)(ii)(A), would state that amounts must be expressed in the increments specified in § 101.9(c)(1) through (c)(7), which includes increments for sodium. One comment said we should permit the use of additional units of measure for dietary ingredients to allow for use of more appropriate units of measure when metric weight is not the most accurate way to express the quantity of the dietary ingredient. The comment gave examples of "colony forming unit" (CFU) for probiotics and enzyme assay units (*e.g.* HUT, PC, SU, ALU) for enzymes. Another comment would amend § 101.36(b)(2)(ii)(A) to state "these amounts shall be expressed in metric or other appropriate units of measure."

(Response) We decline to permit the use of additional units of measure for dietary ingredients. The comment provided the examples of CFUs for probiotics and enzyme assay units for enzymes; however, the broader change suggested in the comment, by including "other appropriate units of measure," would allow for the use of units of measure for dietary ingredients other than just probiotics and enzyme assay units.

We recognize that manufacturers are using a number of different units of measure for probiotics, enzymes, and other dietary ingredients. We need to fully evaluate each unit of measure for dietary ingredients to determine if it is appropriate for use on the Supplement Facts label, and if there are any implications to allowing for the use of such units of measure on the label. Because of the complexity of these labeling concerns, we plan to issue information related to this subject at a later date. We have, therefore, finalized § 101.36(b)(2)(ii)(A) without change.

4. Order of Nutrients Declared on the Label

For dietary supplements, § 101.36(b)(2)(i)(B) specifies that vitamins and minerals must be declared in a specific order on the Supplement Facts label. The proposed rule would add choline to the list of ordered nutrients in § 101.36(b)(2)(i)(B) and that, when declared, choline must follow potassium on the label.

We proposed to amend § 101.9(c)(5) to provide for the voluntary declaration of fluoride, unless a claim about fluoride, in which case fluoride would be mandatory on the label. We inadvertently did not propose to add fluoride to the list of ordered nutrients for declaration on the Supplement Facts label in § 101.36(b)(2)(i)(B).

We did not receive any comments on the proposed addition of choline to the list of nutrients on the Supplement Facts label. Therefore, the final rule adds choline to the list of nutrients in § 101.36(b)(2)(i)(B) and requires it to appear after pantothenic acid on the label because choline is a vitamin and pantothenic acid is the last vitamin in the list of nutrients provided in § 101.36(b)(2)(i)(B). In addition, the final rule specifies that calcium and iron shall be declared after choline on the label because choline will now be declared after pantothenic acid on the label.

As for fluoride, to enable manufacturers to know where to declare fluoride on the Supplement Facts label, we are adding fluoride to the end of the list of nutrients in § 101.36(b)(2)(i)(B) such that, when it is declared, it should be placed below potassium on the Supplement Facts label.

5. Subpopulations

The preamble to the proposed rule (79 FR 11879 at 11947) indicated that, to maintain consistency with the proposed requirements for nutrition labeling of foods in § 101.9, we would revise portions of § 101.36 pertaining to labeling requirements for foods, other than infant formula, that are represented or purported to be specifically for infants 7 through 12 months, children 1 through 3 years, and pregnant and lactating women. The proposed rule would amend § 101.36(b)(2)(iii) to state that the percent of the DV of all dietary ingredients declared under § 101.36(b)(2)(i) must be listed, except that the percent DV for protein may be omitted as provided in § 101.9(c)(7) and that no percent DV is to be given for subcomponents for which DRVs have not been established.

When the percent DV is declared for total fat, saturated fat, total carbohydrate, dietary fiber, or protein, our existing regulations require that a symbol be placed next to the percent DV declaration for these nutrients that refers the consumer to a statement at the bottom of the label that says "Percent Daily Values are based on a 2,000 calorie diet." This statement is only accurate for products meant for children and adults that are 4 years of age and older. In the preamble to the proposed rule (79 FR 11879 at 11947), we explained that the proposed DRVs for total fat, total carbohydrate, dietary fiber, and protein for children 1 through 3 years of age are based on a 1,000 calorie diet, so, when a product that is represented or purported to be for children 1 through 3 years of age contains a percent DV declaration for total fat, total carbohydrate, dietary fiber, or protein, the proposed rule would require, in § 101.36(b)(2)(iii)(D), that a symbol be placed next to the percent DV declaration that refers the consumer to a statement at the bottom of the label that says "Percent Daily Values are based on a 1,000 calorie diet."

The proposed rule also would amend § 101.36(b)(2)(iii)(E) to change the categories of infants and children less than 4 years of age to infants 7 through 12 months of age and children 1 through 3 years of age, and, because we are proposing DRVs for various nutrients for infants 7 through 12 months, children 1 through 3 years, and pregnant and lactating women, amend § 101.36(b)(2)(iii)(F) such that the requirement for an asterisk noting that a DV has not been established would be applicable to foods for these subpopulations only when a DRV has not been established for a nutrient (*i.e.,* for saturated fat, cholesterol, or dietary fiber for dietary supplements that are represented or purported to be for use by infants 7 through 12 months).

We did not receive comments specific to subpopulations and the proposed changes to § 101.36, and so, except as described in our response to comment 474, we have finalized those provisions without change. As discussed in our

response to comment 441, we are using the terminology ''infants through 12 months of age'' throughout § 101.36. As discussed in part II.O.7.a, we also have decided to use the terminology ''pregnant women and lactating women'' rather than ''pregnant and lactating women'' to clarify that the rule is referring to two groups (pregnant women and lactating women) instead of one group.

6. Footnote

The Supplement Facts label can bear a footnote stating that the percent Daily Values are based on a 2,000 calorie diet. In the preamble to the proposed rule (79 FR 11879 at 11947 through 11948), we noted that we intended to modify the footnote on the Nutrition Facts label and to conduct consumer studies related to the footnote on the Nutrition Facts label. We also noted that the footnote for the Supplement Facts label differs from the footnote for Nutrition Facts label, yet we expected that consumers who buy dietary supplements would be more interested in information about the amount of specific micronutrients contained in dietary supplements and would be less focused on the caloric reference value used in determining the percent DV for macronutrients (id.). We said that, based on the results of the consumer study, we would consider whether it is necessary to make corresponding changes to the footnote used on the Supplement Facts label when certain macronutrients are declared, and we invited comment on whether we should change the footnote on the Supplement Facts label to be consistent with the footnote on the Nutrition Facts label.

(Comment 474) One comment said there should be no footnote on the Supplement Facts label. The comment said that consumers do not receive their nutrition solely from a supplement, so, according to the comment, there is no need to refer to total calories. In addition, because all nutrition calculations are being made from the 2,000 calorie total, the comment said that the information provided by the footnote is already standardized across industry, so the footnote is unnecessary.

(Response) We decline to remove the footnote from the Supplement Facts label. Our preexisting regulations, at § 101.36(b)(2)(iii)(D), require manufacturers to declare the footnote ''Percent Daily Values are based on a 2,000 calorie diet'' only when total fat, saturated fat, total carbohydrate, dietary fiber, or protein are declared. The final rule amends § 101.36(b)(2)(iii)(D)) to include added sugars in the list of macronutrients to be consistent with the final requirement to include a declaration for added sugars in the nutrition label. As with the declaration of the footnote statement on the Nutrition Facts label, the footnote statement on the Supplement Facts label provides context for the consumer and enables the consumer to better judge how the nutrients in the supplement contributes towards the total daily diet. Therefore, we decline to remove the footnote statement from the Supplement Facts label.

When the food is purported to be for children 1 through 3 years of age, the final rule requires footnote to state that ''Percent Daily Values are based on a 1,000 calorie diet'' because a 1,000 calorie reference caloric value is used when calculating percent DVs for children 1 through 3 years of age. Therefore, the final rule amends § 101.36(b)(2)(iii)(D) to require the footnote statement ''Percent Daily Values are based on a 2, 000 calorie diet'' on the Supplement Facts label when the percent DV for total fat, saturated fat, total carbohydrate, dietary fiber, protein, or added sugars is declared on the label, and to require the footnote statement ''Percent Daily Values are based on a 1,000 calorie diet'' if the product is represented or purported to be for use by children 1 through 3 years of age and, if the percent DV is declared for total fat, total carbohydrate, dietary fiber, protein, or added sugars.

7. Miscellaneous Comments

Several comments raised other issues regarding dietary supplements and labeling.

(Comment 475) One comment said that the current method of labeling dietary supplements causes confusion regarding which micronutrients, especially vitamins and minerals, are added to a product as opposed to those that are naturally occurring within the product. The comment suggested that the terminology ''naturally occurring'' be used when nutrients are naturally present in ingredients or products, and that other terms, such as ''added,'' be used when ingredients containing micronutrients have been added to a product.

Another comment objected to the nomenclature we proposed for the declaration of certain vitamins and minerals, suggesting the limitations in nomenclature are unconstitutional under the First Amendment (citing *Pearson* v. *Shalala,* 164 F.3d 650 (D.C. Cir. 1999); *reh'g, en banc, denied,* 172 F.3d 72 (D.C. Cir. 1999)) and stating that the nomenclature prevents the dissemination of information helpful to the public in evaluating health implications of supplements. For example, the comment stated that calling tocotrienols vitamin E is not accurate because these forms of vitamin E differ from other forms of vitamin E. The comment also noted that the proposed rule does not distinguish between different forms of vitamin K, selenium, vitamin B_{12}, vitamin B_6, and vitamin B_3 for purposes of identifying on the label the actual ingredient that is contained in a dietary supplement product. The comment suggested that the identification of the actual form of vitamin B_3 that is included in the product is essential because of the physiological differences between these forms. For example, vitamin B_3 could be identified as niacin or niacinamide; and similarly, vitamin B_{12} could be methylcobalamin or cyanocobalamin; vitamin B_6 could be pyridoxal 5-phosphate or pyridoxine; vitamin K could be phylloquinone or menaquione; selenium could be selenomethionine or sodium selenite or selenocysteine. The comment also cited references to suggest selenium in different forms has been reported to have different effects. Furthermore, the comment noted that the name of a nutrient ingredient in a dietary supplement may be a structure/function claim because the form of the molecule determines its function. For example, the comment stated that gamma-tocopherol denotes a particular structure of vitamin E that has a particular function because of its structure.

(Response) With respect to the comment related to added versus naturally occurring micronutrients in dietary supplement products, we decline to revise the rule as suggested by the comment. In dietary supplement products, when terms such as ''naturally occurring'' are used to refer to micronutrients in dietary supplements, they may imply that there is an inherent difference in nutritional quality of the vitamin depending on its source. We are not aware of any evidence that this is the case. Typically, ''added'' nutrients are synthetic forms of the nutrient. As stated in § 101.9(k)(4), a food is misbranded if its labeling suggests or implies that a natural vitamin is superior to an added or synthetic vitamin.

With respect to the comment objecting to the nomenclature we proposed for the declaration of certain vitamins and minerals, the comment seems to misunderstand our requirements for the declaration of vitamins and minerals and for structure or function claims. We provide for the truthful, nonmisleading labeling of

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nutrients in their varying forms on dietary supplements in § 101.36(b) and (d) and § 101.9(c). Our regulation (21 CFR 101.36(b)(2)) provides for the labeling on the nutrition label of dietary ingredients with RDIs such as vitamins or minerals listed in § 101.9(c)(8)(iv), with the exception of vitamin B_3. We discussed, in the preamble to the proposed rule (79 FR 11879 at 11925) and also in part II.M (Reference Daily Intakes for Vitamins and Minerals), the reference intakes for vitamins and minerals listed in the Nutrition Facts and Supplement Facts panels that are identified in § 101.9(c)(8)(iv). The RDIs for vitamins and minerals are based on the IOM RDAs or AIs. In some cases, the RDA is based on the form of a vitamin or mineral recognized to meet human requirements (*i.e.,* the α-tocopherol form of vitamin E) and the AI is based on intakes of a specific form of the vitamin or mineral (*i.e.,* phylloquinone form of vitamin K). With the exception of vitamin B_3, we note that § 101.9(c)(8)(iv) lists the common and usual names of vitamins and minerals. The dietary supplement label requirements at § 101.36(d) provide for labeling of the source ingredient that supplies a dietary ingredient (*i.e.* niacin, vitamin B_{12}, vitamin B_6, vitamin K, and selenium) within the nutrition label in parentheses immediately following or indented beneath the name of a dietary ingredient and preceded by the words ''as'' or ''from,'' *e.g.,* ''Calcium (as calcium carbonate).'' When a source ingredient is not identified within the nutrition label, it must be listed in an ingredient statement in accordance with § 101.4(g). In addition, dietary ingredients, such as menaquinone, that are ''other dietary ingredients'' within the meaning of § 101.36(b)(3) must be declared by their common or usual name when they are present in a dietary supplement in accordance with that section. Thus, the forms of vitamins and minerals contained in dietary supplements such as niacinamide; methylcobalamin or cyanocobalamin; pyridoxal 5-phosphate or pyridoxine; phylloquinone or menaquione; and selenomethionine, sodium selenite, or selenocysteine may be identified, as appropriate, in the Nutrition Facts label or the ingredient statement.

Although we do not recognize the term vitamin B_3 and instead list niacin in § 101.9(c)(8)(iv), the term ''vitamin B_3'' if identified in labeling, other than in the Nutrition Facts label, must be truthful and not misleading. Furthermore, we disagree that we are requiring misinformation by calling tocotrienols vitamin E and lumping these forms of vitamin E together. As we discuss in part II.M, we established the RDI for vitamin E based on α-tocopherol § 101.9(c)(8)(iv). In § 101.36, we provide for dietary ingredients, such as tocotrienols for which we have not established RDI's or DRV's and that are not subject to regulation under paragraph (b)(2) of this section, as ''other dietary ingredients'' in § 101.36(b)(3). If other statements are made about ''other dietary ingredients,'' the statements must be consistent with the all applicable statutory and regulatory requirements.

To the extent the comment suggests that our regulations limit the information about the form of a nutrient on the label, we disagree. Although we have specific requirements related to nomenclature for the nutrient declarations, there are ways to convey the source of the nutrient in labeling, and thus, we do not restrict information about the source of the nutrient, provided the information presented is consistent with our statutory and regulatory requirements.

With respect to the comment that the name of a nutrient may be a structure or function claim, a structure or function claim is described in section 403(r)(6)of the FD&C Act. Such a claim is a statement that describes the role of a nutrient or dietary ingredient intended to affect the structure or function in humans or that characterizes the documented mechanism by which a nutrient or dietary ingredient acts to maintain such structure or function (section 403(r)(6)(A) of the FD&C Act). Gamma-tocopherol is a name for a particular form of tocopherol. While the molecular form of a vitamin may result in a particular function, the name of the form does not describe the role of the dietary ingredient in affecting the structure or function in humans nor does it describe a documented mechanism by which the dietary ingredient acts to maintain such structure or function. Thus, structure or function claims are permitted for dietary ingredients provided they meet the applicable statutory and regulatory requirements for such claims.

(Comment 476) One comment said there is confusion whether nutrient declarations on the Supplement Facts label represent only the added nutrients or the total amount of a nutrient based on analysis of the finished product in products where either micronutrients have been added or botanical ingredients are present that are natural sources of particular micronutrients. The comment suggested we could resolve the issue by ensuring that, where micronutrients are listed on the Supplement Facts and/or Nutrition Facts label, the information reflects those micronutrients that are typically present at the end of the shelf-life period in the finished product, taking into account industry-accepted overages/tolerances.

(Response) The Supplement Facts label provides the nutrition information for nutrients that have a RDI or a DRV as established in § 101.9(c). A (b)(2)-dietary ingredient may only be listed if it is a quantitative amount by weight that exceeds the amount that can be declared as zero in § 101.9(c). We are aware that micronutrients are sometimes added to naturally occurring micronutrients. The value declared on the label should be the value that is supported by data that factors in variability generally recognized for the analytical method used for the finished dietary supplement product for the level involved. We disagree that the label declaration should be based on a shelf-life period because the Dietary Supplement Good Manufacturing Practices regulations do not require an expiration date, shelf-life date, or ''best if used by'' date (see 72 FR 34752 at 34912 and 34856). Therefore, not all products would have a shelf-life date that could be used when determining what the final value should be.

(Comment 477) Several comments opposed decreasing the RDIs for vitamins and minerals because of the impact on the dietary supplement industry. The comments also stated that decreasing the RDIs for vitamins and minerals makes it difficult for consumers to get therapeutic dosages of vitamins and minerals in one supplement.

(Response) We address these comments in part II.M.

8. Compliance Requirements for Dietary Supplements

Compliance for dietary supplements is currently determined in accordance with § 101.9(g)(1) through (g)(8), except that the sample for analysis must consist of a composite of 12 subsamples (consumer packages) or 10 percent of the number of packages in the same inspection lot, whichever is smaller, randomly selected to be representative of the lot. The regulation also says that the criteria on class I and class II nutrients given in § 101.9(g)(3) and (g)(4) are applicable to other dietary ingredients.

The proposed rule would require manufacturers to declare added sugars on the Supplement Facts label under § 101.36(b)(2)(i). It would also require manufacturers to make and keep records to verify the amount of dietary fiber,

soluble fiber, insoluble fiber, added sugars, vitamin E, and folate, under certain circumstances for foods (79 FR 11879 at 11956). The proposed rule, at § 101.9(g)(10) and (g)(11), also would establish recordkeeping requirements for foods that contain a mixture of dietary fiber and added non-digestible carbohydrate(s) that does not meet the definition of dietary fiber, foods that contain a mixture of soluble fiber and added non-digestible carbohydrate(s) that does not meet the definition of dietary fiber, foods that contain a mixture of insoluble fiber and added non-digestible carbohydrate(s) that does not meet the definition of dietary fiber, foods that contain a mixture of naturally occurring and added sugars, foods that contain added sugars that are reduced through non-enzymatic browning and/or fermentation, foods that contain a mixture of *all rac*-α-tocopherol and RRR-α-tocopherol, and foods that contain a mixture of folate and folic acid.

The same records requirements in § 101.9(g)(10) and (g)(11) also should apply to dietary supplements. Therefore, the final rule revises § 101.36(f)(1) to include the recordkeeping requirements for specific nutrients under § 101.9(g)(10) and (g)(11).

Manufacturers of dietary supplements may request an alternative means of compliance or additional exemptions under § 101.36(f)(2) when it is technologically feasible, or some other circumstance makes it impracticable, for firms to comply with the requirements of the regulation. This allowance is the similar to what is made for conventional foods under § 101.9(g)(9). Therefore, the final rule, at § 101.36(f)(2), does not refer to § 101.9(g)(9).

Q. Format

Under our preexisting regulations (see, *e.g.,* § 101.9(d) through (f) and (j)), nutrition information must be presented on food labels in a specific format. The elements of format related to the Nutrition Facts label include such features and graphic design principles as the type style (*i.e.,* font) and size of the type (*i.e.,* point); use of boldface, lines, and bars; arrangement of information in one or more columns; column headings; presence of a footnote and use of a symbol (such as an asterisk) to designate a footnote; and whether nutrition information is listed as a percentage or in absolute (*i.e.,* quantitative) amounts. The elements of format also include the alignment of information; whether indentations are used in listing nutrient data; and the use of white space (or negative space) where no image or text exists. The format may differ from package to package according to the amount of space on the package that is available for labeling, as described and detailed in the relevant sections in this document.

The original format of the Nutrition Facts label was informed by a number of factors, including consumer research that we conducted; consideration of the environment in which consumers typically use the label (*i.e.,* grocery stores); the diversity of consumers (*i.e.,* with respect to education, age, socioeconomic status, etc.) for whom the label is intended; and comments and data received on this issue in response to rulemaking activities conducted in the 1990s. Research studies consistently confirmed that simple formats are easier to comprehend and require less consumer effort than complex information formats. A simple format is one that minimizes clutter and best meets the NLEA requirements that nutrition information should enable the public to readily observe and comprehend such information. In addition, a simple format allows consumers to search for accurate nutrition information with minimum effort, and provides information in a succinct manner that maximizes understanding (79 FR 11879 at 11948).

In the preamble to the proposed rule (79 FR 11879 at 11948), we explained that we were not proposing an extensive reformatting of the Nutrition Facts label. We further explained that we were proposing to make changes based on graphic design principles (such as alignment, consistency, repetition, and contrast), highlight key nutrients and key information, and remove or modify parts of the label to assist consumers in maintaining healthy dietary practices. In brief, we proposed the following changes to the format of the Nutrition Facts label: (1) Increasing the prominence of calories and serving size; (2) reversing the order of the ''Serving Size'' declaration and the ''Servings Per Container'' declaration and increasing the prominence of ''Servings Per Container;'' (3) right-justifying the quantitative amounts of the serving size information; (4) changing the phrase ''Amount Per Serving'' to ''Amount Per ____'' with the blank filled in with the serving size; (5) removing the declaration of ''Calories from fat;'' (6) modifying the presentation of the ''% DV'' information by changing its position to the left of the name of the nutrient on certain labels and separating it from the list of nutrients with a vertical line; (7) declaring ''Added Sugars'' as an indented listing directly beneath the listing for ''Sugars''; (8) declaring the quantitative (or absolute) amounts (in addition to percent DVs) of mandatory vitamins and minerals and, when declared, voluntary vitamins and minerals; (9) requiring dual column labeling under certain conditions; (10) modifying the footnote; (11) requiring that all nutrients not currently highlighted in bold or extra bold type be highlighted in a type that is intermediate between bold or extra bold and regular (*i.e.,* semi-bold) type; (12) adding a horizontal line directly beneath the ''Nutrition Facts'' heading; and (13) replacing the listing of ''Total Carbohydrate'' with ''Total Carbs.'' We also invited comments on other issues related to the Nutrition Facts label format, including the use of an alternative format design or requiring the use of a specific font.

The preamble to the proposed rule also discussed certain modifications to be applied to other label formats to maintain consistency with the proposed Nutrition Facts label. These other modifications would pertain to formats for packages of products that contain two or more separately packaged foods that are intended to be eaten individually (*e.g.,* variety packs of cereals and snacks) or that are used interchangeably for the same type of foods (*e.g.,* round ice cream containers (§ 101.9(d)(13)); formats that apply to subpopulations (§ 101.9(e) and (j)(5)); the simplified format (§ 101.9(f)); the tabular display on packages that do not have sufficient continuous vertical space (§ 101.9(d)(11)(iii)); and the tabular display (§ 101.9(j)(13)(ii)(A)(*1*)) and linear display (§ 101.9(j)(13)(ii)(A)(*2*)) for small packages.

Additionally, in the **Federal Register** of July 27, 2015 (80 FR 44303), we proposed text for the footnotes to be used on the Nutrition Facts label and proposed to require the declaration of the percent DV for added sugars on the Nutrition Facts label. In a separate notice published in the **Federal Register** of July 27, 2015 (80 FR 44302), we reopened the comment period for the proposed rule for inviting public comments on two consumer studies: One using an experimental design methodology (the format study) and one using eye-tracking methodology (the eye-tracking study). The purpose of these studies was to examine the combined effects of most of the changes outlined in the proposed rule in their totality; however, both studies also examined certain individual changes, selected on the basis of priorities and resources available at that time.

1. General Comments

To make a determination about the final format for the Nutrition Facts label, we considered many factors including: Comments we received about the proposed label format in response to our proposed rule (79 FR 11879), the supplemental proposed rule (80 FR 44303) and the reopening of the comment period (80 FR 44302); graphic design principles; and results from consumer research conducted by ourselves and others. This is similar to the approach we took when determining the original Nutrition Facts label formats. At that time, our decisions about format elements drew on information collected from a variety of sources including focus groups and a professional package design firm, in addition to label research conducted by FDA and other organizations (57 FR 32060).

(Comment 478) Several comments stated that neither the results of our consumer studies nor those submitted by outside parties support the proposed label changes and that our proposed changes do not improve consumer understanding of nutrition information on the label over the current label format. One comment said that the proposed format changes do not offer ''enhanced value'' to the consumer that would justify a change from the preexisting label format.

(Response) The consumer studies that we conducted focused mainly on comparing the Current, Proposed, and Alternative formats in their totality. We found that overall consumer preferences, understanding, or perceptions of product healthfulness (as indicated by the label) were comparable among the Current, Proposed, and Alternative label formats. In this final rule, we are making minor changes, such as highlighting certain specific features and characteristics of the label, to enhance the information or for other reasons. Our consumer research provided important information and insights about consumer perceptions, judgments, and understanding that will be useful in informing our future consumer education efforts. We acknowledged in our 1993 nutrition labeling final rule that various considerations (*i.e.,* in addition to consumer research) would bear on the selection of a final nutrition label format. We previously said that an essential criterion would be how well a format conveyed information that Congress expected a nutrition label to provide, such as information that would allow people to decide whether to buy a product or to understand the relative significance of the food in the context of the daily diet (58 FR 2079 at 2115). In the consumer studies we conducted to determine the format for the original Nutrition Facts label, no single format emerged as being superior in every aspect that was investigated. We subsequently worked with graphic design experts to develop the new label, drawing on research that considered not only comprehension, but also legibility and literacy (Ref. 257).

(Comment 479) One comment described a study designed to investigate the extent that consumers are able to quickly notice and understand label information, as they would during grocery shopping (Ref. 258). The study compared consumer reactions to FDA's current and proposed versions of four different Nutrition Facts label formats, each portraying a different food product, so that a total of eight different labels were examined. The current and proposed label formats, and the foods depicted, were: Standard format for single-serve yogurt; tabular format for frozen vegetables; dual-column label for breakfast cereal (per serving and with ½ cup skim milk); and a dual-column label for a multi-serving snack mix package (per serving and per container). The comment recommended that we not implement the proposed changes in format for the Nutrition Facts label because, according to the comment, the study indicated that participants perceived few differences between the current and proposed label formats.

(Response) The results of this study are difficult to interpret because a number of details were not provided. Among other things, the comment did not adequately describe or explain the demographic characteristics of the participants, the statistical methods that were used, how the survey instrument was validated, how the participants were selected and the study was administered, and why 90 percent confidence levels were chosen to indicate significant differences rather than the conventional 95 percent confidence interval. In addition, the manner in which some questions were worded could have affected the responses, and the full range of response options was not presented. Furthermore, the proposed snack mix label appeared to be inconsistent in how the ''per serving'' and ''per container'' values were listed for various nutrients. Although the label indicated ''3½ servings per container'' for some nutrients (*e.g.,* calories, carbohydrates, sodium, protein) the amounts that were listed on the label suggested that there were 4 servings per container, and the amount of dietary fiber shown on the label indicated there were only 2½ servings per container. Therefore, we are not able to rely on the results of this study to inform our decisions regarding Nutrition Facts label formats.

(Comment 480) Several comments said that we should not move forward with the proposed nutrition label format changes without conducting further consumer research.

(Response) We disagree with comments suggesting that we should not finalize this rulemaking until we conduct further consumer research (see, also, our response to comment 6). We considered consumer research studies and public comments, and we also relied on graphic design principles (such as contrast, proximity, alignment, consistency, etc.) in deciding how the various Nutrition Facts label formats should appear in finalizing the requirements for the label format.

2. Increasing the Prominence of Calories and Serving Size

The ability to determine the caloric content of packaged foods is important for all consumers, especially those who are trying to control their total caloric intake and manage their weight. Our preexisting regulations require ''Calories'' to be declared in a type size no smaller than 8 point (§ 101.9(d)(1)(iii)) and highlighted in bold or extra bold type or other highlighting (§ 101.9(d)(1)(iv)). While calorie information is mandatory on the Nutrition Facts label, modifying the Nutrition Facts label to give more prominence to calories may benefit consumers in weight control and maintenance, as noted by the OWG in its final report entitled ''Calories Count'' (Ref. 127).

In the preamble to the proposed rule (79 FR 11879 at 11849 and 11948 through 11949), we explained that the OWG recommended, in part, that we issue an ANPRM to solicit comments on how to give more prominence to calories on the food label. The OWG suggested possible changes to the Nutrition Facts label, such as increasing the prominence of ''Calories'' and ''Serving Size,'' providing a percent DV for calories, and eliminating the ''Calories from fat'' declaration, which may detract from the emphasis on total calories. The OWG recommended that we obtain information on the effectiveness of these options on consumer understanding and behavior related to calorie intake (Ref. 127). In response to the 2005 ANPRM, several comments supported increasing the prominence of calories on the Nutrition Facts label. These comments suggested

various approaches for doing so and pointed out the need for additional research to fully understand the effects of potential label changes on consumer understanding and behavior (Ref. 26).

We considered available data from consumer research and comments received in response to the ANPRMs and conducted our own research on food labels. We tentatively concluded that the proposed changes to the number of calories per serving and the number of servings per container would result in these declarations serving as an anchor to the Nutrition Facts label by focusing the reader's attention to this information and therefore would assist consumers to effectively use this information in the Nutrition Facts label (Ref. 259). The proposed rule would revise § 101.9(d) to increase the type size for ''Calories'' and the numeric value for ''Calories'' and also would require the numeric value for calories be highlighted in bold or extra bold type to draw attention to this information, emphasize the importance of calories on the label, and maintain consistency with the bolded declaration for ''Calories.''

We also expressed a tentative view that the Supplement Facts label should have a format similar to the format being proposed for the Nutrition Facts label with respect to increasing the prominence of information for calories. We invited comment on whether any changes we proposed to the Nutrition Facts label also should be required for certain products with Supplement Facts labels, and if so, under what conditions and for which dietary supplement products should such labeling be required.

(Comment 481) Most comments supported our proposal to increase the prominence of the calories declaration, indicating that giving more emphasis to calories on the Nutrition Facts label would likely benefit consumers in helping them to monitor their caloric intake and make healthier food choices. Several comments suggested that increasing the prominence of calories would help focus consumer attention on their total caloric intake because the information on the label would be more visible, readily accessible, and hard to ignore. Many comments noted that the larger, bolder font would draw attention to the calorie content of the product, encourage consumers to consider this information when selecting a product or deciding how much to eat, and help them to grasp the relative significance of a particular food in the context of their daily diet. Other comments said that increasing the prominence of calories also would help consumers compare products when shopping and perhaps encourage them to pay more attention to labels in general. Several comments pointed out that increasing the type size and visibility of calories would be especially helpful to people with impaired vision, including many older adults and diabetics, and even people with normal vision would benefit if shopping in a dimly lit grocery store. The comments said that, although information about other nutrients is important, information on calories is particularly important because of the prevalence of obesity and the association between obesity and chronic diseases and disabilities. The comments agreed that enlarging the calories information and making it bolder would be an important step, not only in fighting obesity, but also in controlling diabetes.

Although most comments acknowledged the importance of calories and supported increasing the prominence to some extent, many comments opposed declaring the calorie information in a type size substantially larger than that of other information on the label. Many comments expressed concerns that the proposed format overemphasized calories at the expense of other nutrients declared on the label, and several comments suggested that the calorie information was ''disproportionately large'' or consumed too much label space. Other comments included suggestions for improving the overall design and balance of the label by adjusting the relative type sizes for ''Calories,'' the numeric value for calories, and other nutrition information on the label, including the ''Nutrition Facts'' heading. A few comments stated that there was no need to increase the prominence of calories because the Nutrition Facts label already provides calorie information and that increasing the prominence may not provide any additional benefits.

Several comments said that there is no convincing data that enlarging the calorie information would help consumers choose healthier products and that additional consumer research would be essential for determining a format that improves consumer understanding of calorie information in the Nutrition Facts label. One comment pointed out that, although the FDA consumer study cited in the proposed rule failed to demonstrate that increasing the font size for calories lead to healthier choices, we nevertheless decided to proceed with our proposal to increase the prominence of calories on the label. The comment further stated that, because FDA's own consumer research suggested that a larger font size does not improve consumer awareness of the calorie information, we must provide another justification to increase the font size.

Many comments also expressed concerns that overemphasizing calories could have the unintended consequence of suggesting that information about calories is much more important than information about other nutrients appearing on the label. For example, some comments said that the proposed Nutrition Facts label could give the impression that calorie counting is the most important consideration in managing health, when, in fact, reducing the risk of chronic diseases and other health-related conditions goes well beyond caloric intake. Other comments said that consumers might evaluate and compare food or beverage products based solely on their caloric content and choose the option having the fewest calories, without considering the product's total nutrient profile. Consequently, this could inadvertently result in consumers avoiding nutrient dense foods as recommended by the Dietary Guidelines for Americans.

Several comments expressed concerns that making the calorie declaration so prominent could affect consumer use and understanding of other information on the Nutrition Facts label. For example, comments suggested that, because the ''Amount per ____ (serving)'' declaration is relatively small compared to the proposed ''Calories'' and ''____servings per container'' declarations, consumers may mistakenly associate the numeric value for ''Calories'' with the contents of the entire container, rather than with only one serving. Several comments emphasized that consumer research is needed to further investigate formats that would facilitate consumer understanding of this label information and ensure that the format does not result in consumers misinterpreting the calories information. One comment suggested that as part of a consumer test, the ''Amount per ____'' (*i.e.,* serving size) listing and the numeric value for ''Calories'' could be shown in equal type sizes.

(Response) We agree that giving more prominence to calories by increasing the type size and bolding of the ''Calories'' declaration and the numeric value for ''Calories'' would emphasize the importance of calories on the Nutrition Facts label.

We disagree with the comments suggesting it is not necessary to increase the prominence of the calorie declaration or that the numeric value for calories should not be larger than the word ''Calories,'' because, as we explain later in this response, emphasizing this

information has potential benefits to consumers who read the label. However, we agree that the 24 point type size that was proposed for the numeric value for ''Calories'' on most label formats (excluding small packages and dual column labels using the tabular format) could be considered too large and that adequate prominence could still be achieved by slightly reducing the type size. Therefore, the final rule, at § 101.9(d)(i)(iii), requires a type size of 22 point for the numerical value for ''Calories,'' (excluding labels for smaller packages that have a total surface area available to bear labeling of 40 square inches or less) and a type size of 16 point for the word ''Calories'' on all label formats (excluding labels on smaller packages, with a total surface area available to bear labeling of 40 square inches or less and all tabular displays) and highlighting both pieces of information in bold or extra bold type. The requirements for smaller packages require a type size of no smaller than 14 point for the numerical value for ''Calories'' for the tabular display for small packages as shown in § 101.9(j)(13)(ii)(A)(*1*) and the linear display as shown in § 101.9(j)(13)(ii)(A)(*2*), a type size of no smaller than 10 point for the word ''Calories'' for the tabular displays as shown in § 101.9(d)(11)(iii) and (e)(6)(ii) and for the tabular display for small packages as shown in § 101.9(j)(13)(ii)(A)(*1*) and the linear display as shown in § 101.9(j)(13)(ii)(A)(*2*). These type sizes will be sufficiently large to emphasize the importance of calories on the label and draw attention to this information while decreasing the size to address issues raised in the comments as well as accommodating size constraints for packages with a total surface available to bear labeling of 40 square inches or less (see our response to comment 517).

We disagree with the comments suggesting that emphasizing calories would detract from information about other nutrients on the label, or would result in consumers avoiding nutrient dense foods. No evidence was submitted in support of these comments, and we are unaware of any data that emphasizing the calories declaration would encourage consumers to always choose the lower calorie option, result in poor nutritional practices, or lead to adverse health consequences. Although we also are unaware of any consumer studies demonstrating that increasing the prominence of calories information on the Nutrition Facts label would either help or hinder consumer use and understanding of this information, we explained in the preamble to the proposed rule (79 FR 11879 at 11949) that existing data from studies on warning label and drug label formats have demonstrated that increasing the prominence of label information such as warning statements increases consumer attention to such information. Furthermore, the OWG report suggested that we consider increasing the font size for calories on the Nutrition Facts label because of the critical importance of caloric balance in relation to overweight and obesity (Ref. 127). Similar to graphic design principles underlying the appearance of warning labels, increasing the prominence of calories would be expected to draw consumer attention to this information. The OWG report recommend mainitaining a healthy body weight and calorie balance is key factor for managing body weight. The OWG report concluded that obesity is positively associated with adult morbidity and mortality and has become a pervasive and urgent public health problem in the United States. The OWG report also emphasized the medical and health related costs that result from high rates of overweight and obesity. Moreover the 2015–2020 DGA does not alter these conclusions and corroborates these findings. We agree with the OWG report's recommendations and conclusions particularly emphasizing calories, but we are sensitive to concerns about over-emphasizing the calories declaration on the label. An important goal in addressing concerns regarding nutrient density is education. Nutrition education, especially around the Nutrition Facts label should be multifactorial and highlight the importance of calories, but also the other nutrients that can affect health and chronic disease. Therefore, the final rule requires a smaller type size for the number of calories on all labels than what we had originally proposed (*i.e.,* 22 point rather than 24 point for all displays except those for smaller packages), and even further decreased type size (14) requirements are permitted for small packages with a total surface area available to bear labeling of 40 square inches of surface area or less as described in § 101.9(j)(13)(ii)(A)(*1*) and (*2*).

(Comment 482) A few comments expressed concerns that excessively focusing on calories and drawing too much attention to the caloric content of a food product would likely have a negative impact on individuals who are at risk for an eating disorder, or who are already struggling with an eating disorder.

(Response) The comments did not submit data or other evidence to show that eating disorders could be triggered or exacerbated by enlarging the ''Calories'' declaration on the Nutrition Facts label. We are unaware of the existence of such an association and remain convinced that the potential public health benefits of increasing the prominence of ''Calories'' would outweigh the risk of a possible negative impact on individuals struggling with eating disorders.

(Comment 483) One comment stated that, because dietary supplement labels often contain a large amount of information on a small label, increasing the prominence of calories information would likely be difficult because of a lack of space. The comment stated that an increased prominence for ''Calories'' on Supplement Facts labels should be required only if consumption of the dietary supplement would make a major contribution to daily caloric intake (*e.g.,* 50 or more calories per serving). However, the comment noted that, in most cases, dietary supplement products contribute insignificant amounts of calories to the overall diet.

(Response) In the preamble to the proposed rule, we invited comments on whether any of the changes being proposed for the Nutrition Facts label should also apply to products with Supplement Facts labels that list calories and/or other macronutrients (79 FR 11879 at 11949). We did not propose increasing the prominence of calories on labels of dietary supplement products and did not display the calories information in a larger and bolder type size in any of the labels illustrated in the proposed rule in § 101.36(e)(11) and § 101.36(e)(12). We agree with the comment that many dietary supplement products may contribute a negligible amount of calories. Therefore, the final rule does not require that information about calories be displayed in a larger type size or be highlighted in bold or extra bold type or other highlighting on any Supplement Facts labels.

(Comment 484) Several comments pointed out that increasing the font size for ''calories'' and ''serving size'' on the Nutrition Facts label would affect the size of the percentage juice declaration that manufacturers are required to make on juice products. Under § 101.30(e)(2), the percent of juice declaration must be in a height not less than the largest type found on the information panel except that used for the brand name, product name, logo, universal product code, or the title for Nutrition Facts. Because information about ''Calories'' is not included among these exceptions, the type size of the juice declaration would have to be at least as large as the type size of the numeric value for ''Calories.''

Therefore, according to the comments, increasing the size of the ''Calories'' information would mean increasing the size of the percent juice declaration significantly. The comments further suggested that we revise § 101.30(e)(2) to clarify that the percent juice declaration does not have to be larger than the information about ''Calories'' or ''Serving size.''

(Response) We inadvertently omitted the corresponding correction to § 101.30(e)(2) to include ''Serving size,'' ''Calories,'' and the numerical value for ''Calories'' in the list of exceptions for declarations in larger type to avoid requiring a type that would be too large for the declaration of the amount of juice. Therefore, we have made a technical correction in the final rule and revised § 101.30(e)(2) to state that the title phrase ''Nutrition Facts, the declaration of ''Serving size,'' ''Calories,'' and the numerical value for ''Calories'' appearing in the nutrition information must be in easily legible boldface print or type in distinct contrast to other printed or graphic matter, in a height not less than the largest type found on the information panel except that used for the brand name, product name, logo, or universal product code.

(Comment 485) One comment said we should not require the calories information listed on labels of food products intended for infants and young children to have the same prominence as the calories information on product labels intended for people 4 or more years of age. The comment stated that decisions about food choices that are made for infants and young children should not be based on the number of calories per portion, but rather on the overall nutrient profile of the food. The comment explained that, by relying too much on a food's caloric content, parents may inadvertently restrict healthful foods or make inappropriate food choices for their young children and infants. The comment also said that, according to nutrition experts, children in this age range should be encouraged to self-regulate caloric intake and that parents and caregivers should feed children in response to the child's hunger and fullness cues rather than on the basis of a preconceived number of calories they believe the child should consume.

(Response) We agree with the comment that food choices for infants through 12 months of age and children 1 through 3 years of age should focus primarily on a food's overall nutrient profile rather than on the number of calories per serving (Refs. 260–261). The IOM report advocated feeding children in response to their hunger and fullness cues, rather than providing foods for children based on the number of calories in a serving of the product. However, the IOM report also emphasized the importance of parents establishing healthful eating habits for their children early in life. The IOM report stated that children who consume a diet that restricts energy-dense foods high in sugar, fat, and salt, but that is rich in nutrient-dense foods, are less likely to become overweight or obese. Thus, although the IOM report did not explicitly recommend restricting children's foods based on calorie content, it suggested that parents and caregivers should at least be aware of the amount of calories (and other nutrients) in the foods they give their children, especially those over 2 years of age, in order to begin establishing good eating habits.

The comment did not provide evidence that parents would restrict foods or make inappropriate food choices for their young children and infants based solely on the food's caloric content. We acknowledge that parents and caregivers would likely consider a variety of factors when making decisions about what to feed their young children and that increasing the prominence of calories information on the labels of foods intended for young children does not necessarily mean that parents would restrict these foods. Therefore, we do not consider it necessary for the calories information on products for infants through 12 months of age and children 1 through 3 years of age to differ from that required on Nutrition Facts label formats for foods intended for individuals 4 years of age and older. To maintain consistency in label formats, the final rule requires that the calories information on labels of foods intended for infants through 12 months of age and children 1 through 3 years of age be displayed prominently, as indicated in the label mockups shown in § 101.9(j)(5)(i) and (ii).

3. Changing the Order of the ''Serving Size'' and ''Servings Per Container'' Declarations and Increasing the Prominence of ''Servings Per Container''

Our preexisting regulations specify that information on serving size, consisting of a statement of the serving size (§ 101.9(d)(3)(i)) and the number of servings per container (§ 101.9(d)(3)(ii)), must immediately follow the identifying heading of ''Nutrition Facts.'' In addition, ''Serving Size'' and ''Servings Per Container'' must be in a type size no smaller than 8 point (§ 101.9(d)(1)(iii)).

In the preamble to the proposed rule (79 FR 11879 at 11949), we explained that, with respect to the Nutrition Facts label, an important consumer need is to identify the number of servings per container of a packaged food. Therefore, we proposed placing ''Servings Per Container'' above ''Serving Size'' to help consumers find the number of servings per container with less effort than is now needed. We also proposed that listing ''____ servings per container'' with the blank filled in with the actual number of servings directly beneath the ''Nutrition Facts'' heading, and highlighting it in bold or extra bold type, would help increase awareness that the information presented in the Nutrition Facts label does not refer to the contents of the entire package when the label indicates that there is more than one serving per container. We explained that listing ''Serving size'' in the same proximity to where the actual nutrient information is located on the label (rather than directly beneath the Nutrition Facts heading as in our preexisting regulations, § 101.9(d)(3)) would help consumers understand that this nutrient information pertains to the particular serving size that is declared. (According to the graphic design principle of proximity, items that are positioned closer together are perceived to be more closely related (Ref. 262)). Thus, we tentatively concluded that reversing the order of the declarations of ''Servings Per Container'' and ''Serving Size'' would help consumers more readily observe and comprehend the nutrition information appearing in the Nutrition Facts label, allow consumers to search for information with a minimum of effort, and assist consumers in their food purchasing decisions and in maintaining healthy dietary practices. We proposed to redesignate § 101.9(d)(3)(i) as § 101.9(d)(3)(ii), redesignate § 101.9(d)(3)(ii) as § 101.9(d)(3)(i), and to make changes in how the serving size information is capitalized on the label so that no capital letters are used, except for the first letter in ''Serving size.'' We also proposed to require that the declaration of ''____servings per container'' (with the blank filled in with the actual number of servings) be highlighted in bold or extra bold type and be in a type size no smaller than 11 point (except for the tabular and linear displays for small packages) (proposed § 101.9(d)(3)(i)), and that the information for ''Serving size'' be in a type size no smaller than 8 point (except for the linear display for small packages) (proposed § 101.9(d)(3)(ii)).

We did not propose similar changes for serving size information for dietary supplements. In the preamble to the

proposed rule (79 FR 11879 at 11950), we said that, when taking dietary supplements, consumers need to know how much of the product to take (*e.g.,* 1 capsule, 2 tablets, 1 packet) and that this information, which is currently provided in the "Serving Size" line of the Supplement Facts label, is more important for the consumer to know than the number of servings (*e.g.,* 100 tablets) contained in the package.

(Comment 486) Many comments supported changing the order of the "Serving Size" and "Servings Per Container" declarations because the comments felt that this change would make the label easier to read and understand. The comments said consumers would be better able to compare products when shopping and make better buying decisions, which could ultimately lead to improved health for themselves and their families. Other comments suggested that the proposed changes could help consumers understand that nutrition information on the label is based on the serving size, which could increase awareness of the amount of food actually being consumed. In addition, comments said that the proposed change could help consumers monitor their caloric and nutrient intakes, compare products more easily, eat more moderate portions, and more easily grasp the relative significance of a food product in the context of their daily diet.

Other comments said that reversing the order of serving size and the number of servings per container, especially in combination with increasing the prominence of information about calories, would make the relationship between the "Calories" and "Serving size" declarations clearer, lead to a better understanding of the calories information, and improve the flow of the label.

In contrast, several comments opposed changing the order and said we should continue to list "Serving size" above "____ servings per container." The comments suggested that information about a product's serving size was more important than the number of servings per container because the label's information is based on the serving size declaration. Many comments that opposed reversing the order of serving size and servings per container expressed a preference for us to increase the prominence of serving size instead. The comments said that putting the "Serving size" declaration in bold print and increasing its type size would emphasize its importance and increase awareness that the nutrition information on the label is based on the serving size.

(Response) As we explained in the preamble to the proposed rule (79 FR 11879 at 11949), reversing the order in which "Serving Size" and "Servings Per Container" are listed would place the serving size information in closer proximity to where the actual nutrient information is located on the Nutrition Facts label. According to graphic design principles (*i.e.,* the principle of "proximity"), this would increase the perception that the serving size is closely related to the nutrition information that follows directly below it, and thus provide necessary context for helping consumers understand that this nutrition information pertains to the particular serving size that is declared. If the order of the "Serving Size" and "Servings Per Container" declarations was preserved as in our preexisting regulations and as preferred by some comments, the relationship between the nutrition information and the serving size might be less clear. Although some comments suggested that we put the serving size declaration in bold print rather than shift its position, it is unlikely that bold print, alone, would provide the necessary context for helping consumers to understand the association between serving size and the nutrient information because these pieces of information in the preexisting regulation would be lacking in proximity, and the contrast between the "Serving size" declaration and the "Nutrition Facts" heading directly above it would be reduced if both were in a bold or extra bold font. We address the comments concerns regarding increased emphasis of "serving size" instead of "servings per container" in our response to comment 488.

Therefore, the final rule, at § 101.9(d)(3)(ii), requires that "serving size" be placed below "__Servings per container." The final rule also requires the information to be highlighted in bold or extra bold and be in a type size no smaller than 10 point, except the type size must not be smaller than 8 point for the information for small packages as shown in § 101.9(j)(13)(ii)(A)(*1*) and (*2*). Displaying both pieces of information related to serving size adjacent to each other should help consumers understand how the serving size relates to the nutrition information on the label and use the label to plan and maintain healthy dietary practices. It is important for consumers to understand the serving size and realize how it relates to the rest of the label's nutrition information.

(Comment 487) Many comments supported inserting the actual number of servings at the beginning of "servings per container" statement because this could help consumers identify more readily the number of servings in a package and help consumers decide how many people a particular food item could serve or feed. The comments said that consumers would have a better idea of the total number of calories in the package as well as the number of calories they would actually consume if they eat the entire contents of a multi-serving package.

(Response) We agree with the comments, and so the final rule, at § 101.9(d)(3)(i), requires the actual number of servings at the beginning of the "servings per container" statement.

(Comment 488) Many comments agreed that increasing the prominence and visibility of "servings per container" would enable consumers to notice and use this information. The comments further stated that individuals who did not previously or regularly use the label might begin to do so and that increasing the prominence of the "servings per container" declaration would not only be "eye catching" and "hard to ignore," but also would be helpful to people with poor vision or those who shop in dimly lit grocery stores.

Some comments suggested increasing the size and prominence of the "Serving Size" declaration, as well as that of "servings per container." One comment acknowledged that one intention of the proposed rule is to help consumers more easily recognize multi-serving packages, but said there was no valid justification for making the "____ servings per container" information more prominent than the "Serving size" declaration. Another comment suggested that increasing the prominence of both calories and serving size could be especially important on labels of some sugar-sweetened beverages, particularly on products that may contain more than one serving, but are often consumed during one eating occasion.

Several other comments opposed increasing the prominence of "servings per container" because, according to the comments, "serving size" is the more important piece of information. The comments would emphasize "Serving size" in a larger and bolder font. Many comments said that making the serving size information easier for consumers to see and understand was important for properly interpreting the calorie information (in addition to increasing the prominence of "Calories") and is also "what consumers are used to" seeing. Several comments said that the proposed font size of the "____ servings per container" statement was so large

that consumers might mistakenly think that the number of calories listed in the ''Calories'' declaration on the label pertained to the entire package; *i.e.,* to all of the servings that appear in the ''____'' space. Another comment suggested reducing the type size for ''____ servings per container'' to a size smaller than the ''Amount per ____'' statement. One comment suggested that the relative differences in type sizes in the listings for the number of servings per container, the amount per serving, and the numeric value for ''Calories'' could result in consumers mistakenly associating the number of calories with the total package because the ''Amount per ____'' is relatively small compared to the other declarations. One comment said that giving increased prominence to ''Serving size'' would be a reasonable way to implement the recommendations of the OWG's Calories Count report and would be consistent with existing research data suggesting a lack of attention to this listing.

(Response) The comments reflect the need to consider how much emphasis to provide for the ''Serving size'' declaration compared to the ''____ servings per container'' declaration. We agree with the comments that the serving size information was not prominent enough in our proposal and that consumers could potentially associate the calorie and nutrition information on the label with the ''servings per container'' declaration since it was more prominent compared to the serving size declaration. We also agree that the ''servings per container'' declaration should be more prominent and visible than on the preexisting label so consumers will be able to use this information if they consume all or a larger portion of a multi-serving container. Increasing the prominence of the ''Serving size'' information by bolding and slightly increasing the font size will emphasize the importance of the information and, along with its placement, would assist consumers in better understanding how to use the Nutrition Facts label to interpret accurately the calories and nutrient information on the label that is directly below the ''Serving size'' declaration. To provide prominence to ''Serving size,'' however, we need to reduce the prominence of ''servings per container.'' According to graphic design principles (*e.g.,* contrast), alternating a larger and bolder type style with a smaller, regular type style on successive lines of the Nutrition Facts label will provide maximum visibility and optimal highlighting to the information that we wish to emphasize on the label (Ref. 262). Contrast is a graphic design principle that uses opposing elements (such as bolding) to differentiate objects in the same field of view, or to intensify the effect between objects that would otherwise look similar (Ref. 263). Thus, we are providing contrast in the first three lines of the Nutrition Facts label in the final rule (*i.e.,* the Nutrition Facts heading, the ''____ servings per container'' declaration, and the ''Serving size'' declaration) by alternating the use of bold font with non-bold font for this information. We also realize that enlarging the ''____ servings per container'' declaration through bolding may pose space challenges if the word ''about'' is used in this statement, which is allowed under § 101.9(b)(8)(i).

Therefore, the final rule requires that the ''Serving size'' declaration, and the quantitative information associated with this declaration, be listed in a type size no smaller than 10 point (except on labels of smaller packages with a total surface area available to bear labeling of 40 square inches or less and all tabular formats where a type size of 9 point type is permissible due to space constraints) and be highlighted in bold or extra bold type. Additionally, if a product has a ''Serving size'' declaration with too many characters to fit in the provided space allocated for the ''Serving size''declaration, then a type size of 8 point is permissible for any size package (§ 101.9(d)(3)(ii)). To reduce the prominence of the ''____ servings per container'' declaration, we are requiring that ''____ servings per container'' be listed in a regular type in a type size no smaller than 10 point (except on labels of smaller packages with a total surface area available to bear labeling of 40 square inches or less (§ 101.9(j)(13)(ii)(A)(*1*) and (*2*)) where a type size of 9 point is permissible due to space constraints) directly beneath the Nutrition Facts heading, followed directly below by the ''Serving size'' declaration in bolder font.

(Comment 489) One comment referred to a study suggesting that many consumers do not look at serving size information, but otherwise do refer to the Nutrition Facts label and ingredients list, as evidence that the serving size declaration needs to be made more prominent. Other comments suggested that we should more closely review previous consumer research studies or conduct additional studies to determine the effects of displaying ''Serving size'' and ''servings per container'' information more prominently, and determine the potential implications of increasing the prominence and changing the location of the ''____ servings per container'' information on the Nutrition Facts label.

(Response) We disagree with the comment suggesting that many consumers do not look at serving size information, but otherwise do refer to the Nutrition Facts label and ingredients list. The comment apparently misinterpreted a published abstract (Ref. 264) of a study that investigated consumer perceptions and use of the serving size information, ingredient list, health claim information, and the Nutrition Facts label in general, particularly with regards to the extent that each of these impact purchasing decisions. The study, which drew on data from the 2005–2006 and 2007–2008 NHANES, was recently published in its entirety (Ref. 265). In contrast to what the comment said, the abstract stated that the study participants were more likely to use the Nutrition Facts label (in general) and the ingredient list in particular than information about serving size and health claims. In addition, according to data from the NHANES 2009–2010 cycle, approximately 64 percent of respondents (16+ years of age) reported at least ''sometimes'' using the serving size information on the food label when deciding to buy a food product, and 31 percent of the respondents reported that they used the serving size information either ''always'' or ''most of the time'' (Ref. 266).

As for the comments suggesting that we need to evaluate consumer research and conduct further research in regards to switching the order and increasing the prominence of ''Serving size'' and ''servings per container,'' we address these issues in our responses to comments 478 and 480. We also note that we are finalizing the requirement to include, directly below ''Nutrition Facts,'' the ''servings per container'' declaration followed by the ''Serving size'' declaration. As we explain in our response to comment 488, the location of ''Serving size'' to where ''servings per container'' was formerly located places it in closer proximity to the nutrient information that pertains to the serving size of the product.

(Comment 490) One comment said that ''____ servings per container'' is irrelevant information because the nutrition information on the label refers to the amount of nutrients and calories in a single serving. The comment would have the Nutrition Facts label emphasize the size of a serving (*i.e.,* the serving size) rather than the number of servings that are in the container.

(Response) The declaration of ''____ servings per container'' provides important information to the consumer

about how the information on calories and nutrients for one serving of food relate to the entire package of food. Consumers may consume more than one serving and need to know how the portions consumed relate to their total daily dietary intake. Therefore, we decline to revise the rule as suggested by the comment. However, we have revised § 101.9(d)(3) to clarify that both the "____ servings per container" and "Serving size" declarations are components of the serving size information required on the label.

(Comment 491) Other comments opposed increasing the prominence of "__ servings per container" because, in combination with other proposed changes, it would increase the space requirements for the Nutrition Facts label. One comment said that, because of space limitations on the label, we should not require the words "per container" to be included in the "____ servings per container" statement. The comment further said that "per container" is not needed for consumers to identify the number of servings in the package. The comment cited data from an online consumer research study (Ref. 267) to assert that 98 percent of the study participants correctly identified the number of servings per package and the serving size when the label did not include the words "per container," while 92 percent of respondents who viewed the proposed Nutrition Facts label (*i.e.,* "____ servings per container") were able to correctly identify this information.

(Response) We note in our response to comment 488 that we are requiring that "____ servings per container" be listed in a type size no smaller than 10 point (except on labels of smaller packages with a total surface available for labeling of 40 square inches or less, where the type size will be no smaller than 9 point) and in regular font in order to provide adequate contrast to the prominent information displayed directly above and below it (*i.e.,* the "Nutrition Facts" heading and "Serving size" information, respectively). We disagree that the words "per container" should not be required to be included in the "____ servings per container" statement because "per container" would provide context and a frame of reference for the number of servings. Furthermore, the comment did not provide adequate details about its study design, methodology, and statistical analyses, and did not include data that would enable us to appropriately evaluate the survey results. Including the words "per container" would remove any potential ambiguity between servings per container and the serving size information, which would help clarify the number of servings to which the label refers. Although the survey findings reported in the comment indicated that respondents did not need to see "per container" on the label to correctly interpret information about serving size and the number of servings per container, it is difficult to evaluate the results without any data. Therefore, we decline to change our longstanding practice of including "per container" as part of the "servings" declaration, as this information is intended to help consumers accurately identify the number of servings in a package.

(Comment 492) Many comments suggested that we explain that nutrition information is based on the serving size listed in the Nutrition Facts label or conduct an education program to help consumers understand that the label serving size is not a recommendation but is based on actual food intake data. Some comments also asked us to explain the difference between serving size and portion size. One comment stated that, because some consumers use the terms "serving size" and "portion size" interchangeably, we should clarify the label by either: (1) Denoting the serving size provided as a "typical" serving size; or (2) including a footnote to clarify that "the serving size is based upon the amount typically consumed, and is not a recommended portion size." Other comments said it was important to educate consumers that, if one eats more than one serving of a food product, the amount of calories consumed will increase proportionally.

(Response) We recognize the importance of providing consumers with more in-depth information about the meaning of the serving size and intend to make this a key component of our future nutrition education efforts for consumers. However, we decline to revise the rule to add a footnote to the Nutrition Facts label to indicate that the serving size is based on what is typically consumed, rather than what is recommended. Manufacturers can include a truthful and not misleading statement explaining the meaning of serving size elsewhere on the product label.

4. Right-Justifying the Quantitative Amounts Declared in the "Serving Size" Statement

In the preamble to the proposed rule (79 FR 11879 at 11950), we said that we tentatively concluded, based on design considerations, that the label statement for "Serving size" in both household units (*e.g.,* cups, tablespoons, teaspoons, pieces or slices, as explained in § 101.9(b)(5)) and gram amounts must be right-justified on the same line that "Serving size" is listed. Under our preexisting regulations at § 101.9(d)(12), this numerical information is stated immediately adjacent to the "Serving Size" declaration. By keeping the proposed "Serving size" declaration left-justified while right-justifying the corresponding numerical values, the proposed change would create white space on the Nutrition Facts label that would result in a less cluttered appearance, heightened focus and emphasis, and improved readability (Ref. 268). This design feature would provide enhanced emphasis to the information about serving size, allowing this information to be more noticeable and thereby facilitating its access and use by consumers.

(Comment 493) Some comments addressed the issue of right-justifying the quantitative amounts declared in the "Serving size" statement. One comment suggested that moving the serving size information to the right-hand side of the Nutrition Facts label would help emphasize the information, create white space leading to a less cluttered appearance, and would allow the eye to "flow across the information." Another comment said that the proposed change would make it easier for readers to find the values for calories, serving size, number of servings per container, and percent Daily Values if all of these values were consistently placed in the same right-hand side of the label.

One comment opposed to right-justifying the serving size quantitative information on the Supplement Facts label. The comment said that because the "Serving size" declaration must be left-justified, the quantitative information for serving size should appear near this declaration, rather than on the other side of the panel where it would be separated by a large white space. The comment added that this may be a particular concern for dietary supplement products that use dual column labeling (*e.g.,* with columns for "Per Serving" and "Per Day").

(Response) Keeping the "Serving size" declaration left-justified, while requiring the corresponding numerical value be right-justified, provided that adequate space is available, will make this information more noticeable and facilitate its access and use by consumers. Although we did not propose to right-justify quantitative amounts in the "Serving size" declaration in the Supplement Facts label, we agree that it would not be appropriate to do this. The "Supplement Facts" title in the Supplement Facts label requires more

space than the ''Nutrition Facts'' title in the Nutrition Facts label and (unless impractical) must span the full width of the label (§ 101.36(e)(1)). Also, the Supplement Facts label is less likely than the Nutrition Facts label to be situated on the narrow side panel of a package. Therefore, because Supplement Facts labels are often wider than Nutrition Facts labels, right-justifying the serving size amount might leave too much white space between the words ''Serving size'' and the quantitative amount. It may not be apparent on some Supplement Facts labels that the quantitative amount per serving listed on the far right side of the label would refer to the serving size declaration, which would be left-justified. With dietary supplements in particular, it is important that consumers understand the serving size unit (*e.g.,* 1 tablet, 1 capsule) to minimize the possibility of taking an excessive amount of the product. The serving size amount also is important so that consumers can understand and follow instructions on dietary supplement labels for the suggested use of the product, which explain how, when, or how much of the product to take daily and (if applicable) the amount not to exceed. Therefore, the final rule only requires that quantitative amounts declared in the ''Serving size'' statement be right-justified on Nutrition Facts labels, provided that adequate space is available, and not on Supplement Facts labels.

5. Changing the ''Amount Per Serving'' Statement

Our preexisting regulations require the Nutrition Facts label to include a subheading designated as ''Amount Per Serving'' and to separate this subheading from the serving size information by a bar (§ 101.9(d)(4)) and highlight the subheading in bold or extra bold type or other highlighting (§ 109(d)(1)(iv)). The proposed rule would change the ''Amount Per Serving'' declaration to ''Amount per _____'', with the blank filled in with the actual serving size expressed in household units. We also proposed increasing the type size of this information and, to heighten contrast with the calories information, using semi-bold rather than bold or extra bold highlighting. We explained, in the preamble to the proposed rule (79 FR 11879 at 11950), that these changes would make it easier for label users to understand what the nutrition information in the Nutrition Facts label refers to, because it would eliminate the need to first locate the ''Serving size'' declaration to see what the serving size unit is. Because studies suggest that consumers often find serving size information difficult to interpret (Ref. 9) we stated that specifying the actual serving size in the ''Amount per _____'' declaration would likely help consumers to more readily observe and comprehend the nutrition information that is displayed in the label.

(Comment 494) Some comments supported the proposed change and said that replacing ''Amount Per Serving'' with ''Amount per _____'' would reinforce the concept of serving size and help people realize how many calories are actually in a serving of the product. One comment said it was reasonable for the label to include duplicate information (*i.e.,* in both the ''Serving size'' and ''Amount per _____'' declarations) about what constitutes a serving because it is important for consumers to understand that the nutrition information on the label is based on the serving size. Another comment suggested that both the ''Serving size'' and ''Amount per _____'' declarations should be bolded to increase their visibility.

Many comments disagreed with the proposed change and said it would make the serving size information repetitive, create unnecessary clutter, and impose additional space constraints on the label. One comment said that including duplicative information about serving size would be distracting and ''slow down'' the comprehension process, especially if the serving size is listed as a fraction (*e.g.,* ⅔ cup). Another comment suggested that listing the serving size in the ''Amount per _____'' statement is unnecessary because our proposal to reverse the order of ''Serving size'' and ''Servings Per Container'' and make the ''____ servings per container'' information more prominent already allows the serving size to be more easily identified. The comment said that only the ''Serving size'' declaration should be used to indicate the amount of food contained in a serving, and that doing so would maintain consistency with the current Nutrition Facts label.

Another comment suggested improving the clarity of the label by moving the ''Amount per _____'' declaration directly above the list of percent Daily Values, listing the serving size after ''Calories '' (*i.e.,* ''Calories per _____''), and using the same type size for the ''Serving size'' and ''Amount per _____'' declarations. Another comment said that changing ''Amount Per Serving'' to ''Amount per _____'' should be voluntary for dietary supplement labels, but if the change is made mandatory, then manufacturers should have the option of using the abbreviation ''Amt Per _____'' on Supplement Facts labels when extra space is required for the quantity statement (*e.g.,* ''2 capsules'').

(Response) We recognize there are multiple viewpoints and potential advantages and disadvantages with respect to listing the actual serving size in the blank space of the ''Amount per _____'' declaration. We acknowledge that inserting the serving size in the blank space would essentially repeat the value for serving size that is listed directly above this statement. We further agree that this information would be duplicative and add to the amount of numerical information already present on the label. Therefore, we will retain the preexisting requirement to declare ''Amount per serving'' directly above the ''Calories'' declaration rather than finalize a change to declare ''Amount per _____'' with the blank filled in with the actual serving size expressed in household units. We also will retain the preexisting requirement to list ''Amount per serving'' in bold or extra bold type or other highlighting and in a type size no smaller than 6 point rather than finalize a change in type size and contrast.

With respect to the comment that said changing ''Amount Per Serving'' to ''Amount per _____'' should be voluntary for dietary supplement labels, we did not propose this change for the Supplement Facts label. Consequently, there is no need to provide the option of using the abbreviation ''Amt Per _____'' on Supplement Facts labels as the comment requested.

6. Declaration of ''Calories From Fat''

The proposed rule would eliminate the requirement for declaring ''Calories from fat'' on the label.

Most comments supported removing the requirement for declaring ''Calories from fat,'' and we discuss those comments in part II.E.1.

7. Presentation of Percent DVs

Our preexisting regulations at § 101.9(d)(7) establish the format for listing nutrients with DRVs on the Nutrition Facts label, including the quantitative amount by weight and percent DV. The preamble to the proposed rule (79 FR 11879 at 11950 through 11951) explained that, when we established the requirements for percent DV declaration, we considered that the information would help consumers evaluate the nutrient characteristics of a single product (*e.g.,* how high or low a particular product is in certain nutrients or the extent to which it contributes toward daily nutritional goals) and help

consumers make choices between products. We also explained that consumer research back in 1992 indicated that the percent DV information improved consumers' abilities to make correct dietary judgments about a food in the context of a total daily diet and helped consumers to verify the accuracy of front panel claims (*id.*).

The proposed rule would use ''% DV'' rather than ''% Daily Value'' as the column heading above the nutrient listings to provide consistency among the different label formats and to maintain the alignment of this heading over the DV column. For most labels, the proposed rule also would list percent DVs in a column to the left of the names of the nutrients and their quantitative amounts, with a thin vertical line separating the % DV column from the list of nutrients. On dual column labels and on labels using the aggregate display, we proposed to list the names of nutrients to the left of the % DV columns and the quantitative (weight) amounts of each nutrient to the right of the % DV column, to use thin vertical lines to separate the information in the ''% DV'' column from the information in the column containing the quantitative weights, and to use the same style of thin vertical lines to separate each of the dual columns and aggregate display columns from each other.

We also invited comment on alternative terms that may be more readily understandable than Daily Value, such as Daily Guide or Daily Need; whether the word ''percent'' (or the % symbol) needs to precede whatever term is used in the column heading where the percent DVs are listed or if this would be redundant because the ''%'' symbol is already included next to the numerical values listed in this column; and the appropriate placement of percent DVs in the labeling of foods for infants 7 through 12 months, children 1 through 3 years of age, and pregnant and lactating women (*id.* at 11961).

(Comment 495) Some comments supporting our proposal said that moving the percent DVs to the left would draw attention to this information and help people realize its importance. Some comments said that, because we read from left to right, people would be less likely to skip over the percent DVs. Furthermore, because the information would be more noticeable, consumers might find it more quickly and use it more often to judge the percent DV of a specific nutrient and to compare products when shopping, leading to healthier food choices. Other comments said that shifting the percent DV column to the left would be ''eye catching,'' create a cleaner design, and make the label more logical, better organized, and easier to read and comprehend. It also would improve the simplicity and visual clarity of the label, as recommended by the IOM.

Many comments that opposed placing the percent DV column on the left side of the label said that, because we read from left to right, consumers would see the percent DV before knowing to which nutrient the value referred. The comments said it is more logical to list an item first and then its value. Some comments said that moving the percent DV information to the left of the nutrient name would be counter-intuitive and confusing to consumers. One comment included data from a study it had commissioned; the study indicated that, when the percent DV was on the left side of the label, there was no advantage in consumer comprehension of this information. The study found that a higher percentage of respondents answered a question about Daily Values correctly when the percent DV information was on the right versus the left side of the label (Ref. 269). Another comment noted that the proposed label would be awkward to read because consumers would need to first find the name of the nutrient in the middle of the label.

Several comments agreed with the concern we expressed in the preamble to the proposed rule, that giving more prominence to the percent DV by listing it first could potentially make the Nutrition Facts label appear less user-friendly particularly to frequent users who are accustomed to its current format and could draw attention away from nutrients that do not have a DV (79 FR 11879 at 11951). Another comment said that shifting the percent DV to the left could hinder, rather than assist, individuals with lower levels of health literacy and numeracy in understanding the label.

Several comments said that moving the percent DV information to the left might cause layout problems for certain formats, such as dual-column labels, because of the difficulty in aligning the column headings with the information in the columns, and in differentiating the columns. Other comments expressed concerns that placing percent DVs on the left would be distracting because consumers are mainly interested in the quantitative values of nutrients and tend to look for that information rather than the percent DVs. Other comments said that increasing the focus on percent DVs would be misguided because the percent DVs are not relevant to people who do not eat 2,000 calories per day; moving the percent DVs to the left would make the label look ''foreign'' and would be an unnecessary change having no benefits; and shifting the location of the percent DVs would not help consumers understand the information any better than they currently do. Many comments said that, because people are generally confused by the meaning of percent DV and do not know how to properly use this information, percent DVs should not be given a more prominent placement on the left side of the Nutrition Facts label. Several comments said it was premature to shift the percent DVs to the left based solely on theoretical design principles, and that we should not do this unless research data become available demonstrating that this change would assist consumers in maintaining healthy dietary practices.

(Response) We acknowledge that the conventional way to display data would be to list the percent DV after the name of the nutrient, as shown in the preexisting Nutrition Facts label format, and that shifting the percent DVs to the left might present layout challenges with certain formats. We also note that the results of our consumer research study were equivocal, as we found that no significant benefit was achieved by shifting the percent DV column to the left side of the Nutrition Facts label (Ref. 270).

We have no evidence that the placement of the percent DV information on the left would result in less comprehension by consumers who do not understand the meaning of percent DV, as suggested by some comments. Nevertheless, we have reconsidered how percent DV should be presented and have decided to retain the preexisting requirement to list the percent DV information on the right side of the label.

We anticipate that an increased focus on percent DV through the introduction of a new footnote and enhanced consumer education efforts could help consumers who currently have some difficulty understanding percent DV become more comfortable using the percent DV information. Furthermore, we may study this issue, and other issues involving the DV, in the future.

(Comment 496) Several comments suggested that the term ''Daily Need'' would be more helpful to consumers than ''Daily Value.'' Another comment suggested using the term ''Daily Requirement'' because it would be ''more in keeping with a DRV calculation.'' The comment cautioned that the term ''Need'' may have a

negative perception because it conveys a ''personal tone'' and therefore may be seen as prescriptive or patronizing. An additional comment suggested using ''% Ref'' instead of ''% DV.''

(Response) In the preamble to the proposed rule, we said that we had previously provided our rationale for choosing the term Daily Value in the format final rule (58 FR 2079 at 2124, January 6, 1993) and had explained why we considered ''need'' and ''requirement'' to be misleading terms that might complicate nutrition education efforts. Although one comment suggested the use of the term ''% Ref.'' (which we interpret as meaning % Reference) instead of % DV, the comments, in general, did not suggest alternative terms or provide data or information to support why an alternative term would be more appropriate or preferable. Thus, we continue to believe that the term Daily Value is generally understood by consumers to be a point of reference (see 58 FR 2079 at 2125) and will continue to use Daily Value as an appropriate single term to refer to all reference values in the Nutrition Facts label.

(Comment 497) Many comments opposed the use of the abbreviated term % DV, and suggested that spelling out the term Daily Value would be clearer and easier to comprehend, eliminate possible confusion about the meaning of DV, and not require an explanatory footnote. Some comments stated that, while abbreviating Daily Value would save space, the abbreviation would not be helpful if consumers did not understand the abbreviation, especially when consumer research has shown that the term Daily Value is not well understood. One comment noted that if ''% Daily Value'' was abbreviated to ''% DV,'' we might replace a concept that is already obscure with a shorthand designation that would be even more obscure to consumers.

Another comment suggested that consumer research is needed to evaluate the impact that changing % Daily Value to % DV would have on consumer use and understanding of this information. Some comments supported using ''%'' rather than spelling out ''percent'' because, according to the comments, it would decrease the amount of clutter on the label, and the term ''percent'' requires more label space without providing additional information or benefits to consumers. Another comment questioned whether either ''percent'' or the ''%'' symbol should be used on the label because the comment said that many consumers have difficulty understanding the concept of percent.

(Response) We acknowledge that the term % DV is spelled out on most labels (with the exception of some small packages) and therefore the term ''% Daily Value'' should be familiar to consumers. We also acknowledge that it would be desirable for the Nutrition Facts label to be able to ''stand alone'' as a source of information to assist consumers in maintaining healthy dietary practices, and that the label should be self-explanatory insofar as possible. By spelling out the words Daily Value instead of abbreviating them, the meaning of the nutrition information presented on the Nutrition Facts label would be less ambiguous to consumers, alleviate the need to explain the abbreviation, and improve the ability of the label to stand alone. Therefore, the % Daily Value, rather than % DV, should be used as the column heading for most formats if space is available. In order to provide flexibility to manufacturers when there are space constraints on packages and to facilitate alignment of the % Daily Value column heading with the nutrient information listed beneath it, particularly on formats in which there are multiple columns of information, we are retaining the provision in our preexisting regulations (§ 101.9(d)(6)) that allows for the substitution of ''Percent Daily Value,'' ''Percent DV,'' or ''% DV'' for ''% Daily Value.''

With respect to whether consumers may have difficulty understanding the concept of percent, our public education program will help consumers understand how to use the percent DV information and become more comfortable with the concept of percent. We will continue to use percentages on the Nutrition Facts label for presenting nutrition information because it is useful for assisting consumers in maintaining healthy dietary practices.

(Comment 498) One comment requested clarification with regards to how the percent DV information should be displayed for the nutrients of public health significance when these nutrients are listed either vertically or horizontally in two columns (*i.e.,* the side-by-side arrangement), as permitted in § 101.9(d)(8). The comment said there was a discrepancy in how we described the vertical arrangement of nutrient information for vitamins and minerals in § 101.9(d)(8) and how this information was displayed in the label format shown in proposed § 101.9(d)(12). The comment further suggested that the phrase ''or may be listed in two columns'' should be clarified, particularly with regards to the placement of the nutrient name, the % Daily Value, and the quantitative amounts, and that an example of this label would be helpful.

(Response) The description of the vertical array of vitamins and minerals in § 101.9(d)(8), which the comment said was inconsistent with the associated mockup because the percent Daily Values were listed in parentheses in the regulation, was not meant to be a literal description of what was shown in the label mockup in proposed § 101.9(d)(12). However, we agree with the comment that the phrase ''or may be listed in two columns'' needs to be clarified, particularly with regards to where the percent Daily Values and the absolute amounts are displayed relative to the names of the respective vitamins and minerals. Therefore, we have now stated in § 101.9(d)(8) that the name of the nutrient will be listed first, followed by the absolute amount and then by the percent Daily Value (which will be listed to the right of the absolute amount and without parentheses). Furthermore, as the comment suggested, we have provided a mockup showing the horizontal (*i.e.,* side-by-side) display of the vitamins and minerals in § 101.9(d)(8). However, we also note that mockups are provided as examples of labels, and are meant to serve as illustrations rather than as indications of specific requirements. We have not provided mockups of all possible types of labels and we did not intend to state literally in the regulation what was shown in the various label mockups.

8. Placement of ''Added Sugars''

The proposed rule would require the declaration of added sugars as an indented line item underneath the declaration of total sugars on the Nutrition Facts label. In the **Federal Register** of July 27, 2015 (80 FR 44303), we issued a supplemental proposed rule that would, among other things, establish a DRV of 10 percent of total energy intake from added sugars and require the declaration of the percent DV for added sugars.

We did not receive any comments regarding the indentation of the added sugars declaration. We discuss the requirements for the added sugars declaration in part II.H.3.

9. Declaration of Absolute Amounts of Vitamins and Minerals

The proposed rule would require the declaration of quantitative amounts for all vitamins and minerals listed on the Nutrition Facts label (except on labels of smaller packages with a total surface area available for labeling of 40 square inches or less as described in § 101.9(j)(13)(ii)(A)(*1*) and (*2*)), in addition to maintaining the current

requirement of declaring percent DVs. Because of space limitations, we proposed to require only the percent DV for vitamins and minerals (other than sodium) on labels of foods in small or intermediate-size packages having a total surface area available to bear labeling of 40 or less square inches. As we explained in the preamble to the proposed rule (79 FR 11879 at 11928 through 11929), comments received in response to the 2007 ANPRM, as well as the 2003 IOM report (Ref. 219) supported declaring both the absolute amounts of mandatory and voluntary micronutrients on the Nutrition Facts label in addition to the percent DVs (when they exist). Among other reasons, the IOM report said that listing absolute amounts of all vitamins and minerals would make the Nutrition Facts label internally consistent and more aligned with the current requirements of the Supplement Facts labels (§ 101.36(b)(3)(ii) and (iii)).

We also considered previous research which indicated that both consumers and health professionals have difficulty understanding how percent DVs relate to the absolute amounts of nutrients listed on the Nutrition Facts label (Ref. 239). The previous research indicated that physicians, dietitians, and other health professionals found it easier to refer to absolute amounts of nutrients rather than to the percent DVs when advising patients. The results suggested that declaring both the absolute amount and the percent DV would improve understanding of the label.

(Comment 499) Many comments agreed that we should require the declaration of absolute amounts of all vitamins and minerals on the Nutrition Facts label. Some comments said that people, especially those with low numeracy skills, have difficulty understanding the concept of ''percentage'' (such as percent DV) and would prefer using nutrition information expressed in absolute amounts rather than in percentages to plan diets. The comments also suggested that people who want to follow a health professional's nutrition guidance, such as advice to consume a specific amount of a nutrient (*e.g.,* 500 mg calcium/day), would find quantitative amounts on labels to be more useful than the percent DVs.

Other comments from registered dietitians said they perceived percent DVs to be confusing and cumbersome and preferred to use absolute amounts of nutrients when counseling clients on how to use the Nutrition Facts label to build a healthy diet, compare food products, and establish dietary goals.

In contrast, many comments expressed concerns that declaring absolute amounts of all vitamins and minerals, in addition to the percent DV, would make the label more confusing, cluttered, and difficult to read. The comments said that listing quantitative amounts of all vitamins and minerals would take up valuable label space and add complexity to the label without providing any tangible benefits to consumers. Several comments said that the percent DV listing already provides consumers with the information they need for choosing foods for a healthy diet, so it is not necessary to also list the absolute amounts for all nutrients on the Nutrition Facts label. The comments questioned whether consumers would understand how to use absolute amounts in conjunction with the percent DV and said there was little evidence that declaring absolute amounts on the Nutrition Facts label would help consumers maintain healthful dietary practices. Some comments expressed concerns that, because consumers in general are not familiar with metric system units such as grams, milligrams, and micrograms or the relative magnitude of differences between these units, they may not realize that a quantitative weight listed as a large number, but expressed in micrograms, can actually represent a small amount of the nutrient. Another comment said that, because some high DVs are based on small quantitative amounts and some small DVs are based on high quantitative amounts, the quantitative information could be confusing to consumers.

(Response) In the past, we have stated that we must be selective with regard to the information we require to be listed on the label and that not all vitamins and minerals are of equal public health significance (58 FR 2206 at 2107). We have limited the mandatory declaration of vitamins and minerals to those of particular public health significance. These vitamins and minerals include vitamin D, calcium, iron, and potassium, which are ''shortfall'' nutrients in the general U.S. population that are often consumed in inadequate amounts. In addition, we are requiring the absolute amount for folic acid in mcg to be declared when folic acid is added as a nutrient supplement or claims are made about the vitamin on the label or in labeling of foods (§ 101.9(c)(8)(ii) in the final rule).

As we stated in the preamble to the proposed rule, research suggests that consumers and health professionals have difficulty understanding how percent DVs relate to the absolute amounts of nutrients (79 FR 11879 at 11928 through 11929). We recognize that some consumers, particularly those with low numeracy skills, may be better able to understand and use the listed quantitative amounts of nutrients (*e.g.,* milligrams of calcium) on the label when making dietary choices, rather than relying solely on the percent DV, because they would need to know the calculation for converting percent DV to milligrams. Thus, although some comments would not list absolute amounts because (according to the comments) the percent DV already gives consumers the information they need for choosing foods for a healthy diet, the percent DVs and absolute amounts, particularly for nutrients of public health significance, are useful because consumers receive information on the recommended intake of these vitamins and minerals in quantitative amounts (*i.e.,* the advice is given in milligrams, micrograms, or International Units) through public sources and from health professionals (Refs. 219, 271–272). Furthermore, folic acid intake is related to the risk reduction of neural tube defects, and is generally provided in terms of mcg of folic acid. By requiring the mandatory declaration of folic acid as a quantitative amount by weight in mcg, when folic acid is added or when a claim is made about the vitamin in labeling, women of childbearing age can gain a better understanding of the unique contribution that synthetic folic acid from food provides in reducing the risk of neural tube defects and will have the information they need to improve their ability to adhere to nutrition recommendations with respect to folic acid.

Thus, requiring both the quantitative amount and the percent DV will help to ensure that consumers are fully informed about the content of these products, similar to how these nutrients are declared in dietary supplement product labeling (56 FR 60366; November 27, 1991). Nevertheless, we have decided not to include in the final rule the proposed requirement to include the declaration of absolute amounts for all vitamins and minerals. We clarify, in § 101.9(c)(8)(ii), that the declaration of voluntarily declared vitamins and minerals listed in paragraph (c)(8)(iv) may include the quantitative amount by weight and percent of the RDI. We also revised the preexisting requirement in § 101.9(c)(8) to remove the requirement that the declaration for vitamins and minerals include a statement of the amount per serving as a percent DV. A requirement to compel absolute amounts for all vitamins and minerals could make it

difficult for consumers to use and read the label, particularly on fortified foods such as cereals where many vitamins and minerals may be listed. In addition, the public health need among the general U.S. population is not as great for listing quantitative amounts for voluntary vitamins and minerals, such as thiamin, riboflavin, or niacin, because deficiencies of these vitamins are rare and because enriched bread, rolls, and buns must be fortified with these nutrients. Requiring the declaration of absolute amounts of nutrients of public health significance, and folic acid when added as a nutrient supplement or claims are made about the vitamin, while providing voluntary declaration of absolute amounts for other vitamins and minerals, will provide manufacturers with flexibility in assessing how much voluntary information to provide on the Nutrition Facts label without creating unnecessary clutter. However, if one of these other vitamins or minerals is added as a nutrient supplement or there is a claim made about it, the manufacturer must include a declaration of the nutrient as a percent DV, or alternatively, as a quantitative amount by weight and percent DV (§ 101.9(c)(8)(ii) in the final rule).

With respect to the comment expressing concern that quantitative information could be confusing to consumers, the comment discussed a situation where a product that contains 100 percent DV for vitamin D and lists only 20 mcg (a ''low'' amount) on the label also contains 5 percent DV for potassium, which would correspond to an absolute amount of 235 mg (a ''high'' amount). However, only two of the four nutrients (vitamin D and potassium) are new nutrient declarations under the final rule, and we expect consumers to become familiar with these nutrients as part of the new label. Vitamin D is a shortfall nutrient that many health professionals discuss with their clients or patients as part of a healthy dietary intake. As noted elsewhere in part II.N.4, vitamin D must be listed in micrograms and may be listed voluntarily in International Units. In addition, although only the percent Daily Values for calcium and iron are currently listed on the Nutrition Facts label, consumers who take these nutrients as dietary supplements may be familiar with the corresponding quantitative amounts because these must be declared on Supplement Facts labels. Furthermore, the Nutrition Facts label has included metric units since its inception in 1993, so consumers have had considerable exposure to metric units such as grams and milligrams. To the extent consumers are less likely to be familiar with ''micrograms'' (mcg), we anticipate that consumers will become increasingly familiar and comfortable with this metric unit and others on the Nutrition Facts label. We plan to address the different nutrients of public health concern and their units of measure as part of our education efforts aimed at enhancing consumer understanding of the label.

(Comment 500) Some comments said that for people who have special dietary requirements because of a medical condition, such as chronic kidney disease, the percent DV by itself may be inadequate for making decisions about food selections (*e.g.,* kidney patients who monitor their phosphorus intake would find the phosphorus content expressed in milligrams to be more useful than the % DV of phosphorus).

(Response) While the Nutrition Facts label information has never been, nor is it now, targeted to individuals with acute or chronic disease, consumers may be able to use quantitative information on the label to follow advice they have received from a health care professional concerning their conditions (see part II.B.2).

(Comment 501) Several comments questioning the need for declaring absolute amounts of vitamins and minerals on the Nutrition Facts label said that people who meet their nutritional needs through conventional foods are less likely to be interested in quantitative amounts of vitamins and minerals compared to those who use dietary supplements to supplement their diets with specific amounts of such nutrients. The comments said that labels designed for conventional food products and for dietary supplements are not necessarily analogous because the two types of products have different purposes as reflected in their nutrient composition; *e.g.,* nutrient levels in dietary supplements are often much higher than those in foods and beverages. The comments also noted that, because there is a greater potential for toxicity resulting from the use of dietary supplement products due to overconsumption compared to conventional food products, it is important that nutrient levels on Supplement Facts labels be expressed in absolute amounts so that this information is plainly visible to consumers.

(Response) Requiring the absolute amounts of vitamins and minerals for the nutrients of public health significance and folic acid under the circumstances previously described will help ensure that consumers are fully informed about the content of conventional foods and will achieve parity in labeling for nutrients of public health significance in conventional foods and dietary supplements. We do not consider issues related to potential greater toxicity from consumption of nutrients in dietary supplements to negate the benefits of also providing for conventional foods the information on absolute amounts for these particular nutrients of public health significance that are considered shortfall nutrients.

Requiring absolute amounts of vitamins and minerals of public health significance and folic acid under the circumstances previously described to be listed on the Nutrition Facts label will make it easier for both consumers and health professionals to understand and use the Nutrition Facts label and help consumers in maintaining healthy dietary practices. Furthermore, consumers can use the information to obtain these shortfall nutrients primarily through healthy eating patterns containing nutrient-dense conventional foods, as recommended by the DGA (Ref. 28).

(Comment 502) Several comments expressed concerns that requiring the absolute amounts of all vitamins and minerals to be listed on the Nutrition Facts label would be problematic because FDA's established rounding rules only apply to percent DV declarations, and the proposed rounding rules for declaring quantitative amounts of vitamins and minerals are not clear. The comments said that different products having the same absolute amounts of a nutrient listed on the label may have different percent DVs associated with that nutrient due to rounding. Some comments also said that two different products having the same percent DV for a nutrient may declare different absolute amounts for that nutrient, which would lead to consumer confusion. In addition to such discrepancies, several comments said it is not feasible to require absolute amounts of vitamins and minerals to be listed because analytical assays for obtaining this information lack the necessary precision, resulting in considerable variability in results from assay to assay. Other comments said that levels of nutrients in foods and food products are naturally variable and due to this variability, declaring absolute amounts would imply greater precision than is currently required for the declaration of the percent DV. The comments also said it would be particularly difficult and costly to obtain information on vitamin D levels because this information was not

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previously required for most conventional food products.

(Response) The quantitative amount of sodium has always been required to be declared on the Nutrition Facts label, and dietary supplement products have required weight amounts to be declared since 1993. Rounding rules for the Nutrition Facts label have been established for potassium (§ 101.9(c)(5)) and for other vitamins and minerals (§ 101.9(c)(8)(iii)) in the Nutrition Facts label and for vitamins and minerals declared on labels of dietary supplements (§ 101.36(b)(2)(ii)(B) and § 101.36(b)(2)(iii)(B)). We discuss this topic further in part II.M.6. To declare the percent DV for vitamins and minerals on the Nutrition Facts label, manufacturers should already have information about the levels of nutrients in their products. Such information also can be obtained through laboratory analysis or by consulting standard nutrient databases, such as the USDA Nutrient Data Lab Standard Reference (*http://www.ars.usda.gov/Services/docs.htm?docid=8964*). Substituting vitamin D and potassium for vitamin A and vitamin C for the nutrient analysis should not result in a significant difference in cost to the manufacturer. Furthermore, we are not aware of problems in obtaining quantitative data related to variability and precision. Manufacturers already must address these issues to comply with the preexisting nutrition labeling regulations.

(Comment 503) One comment included the results of a consumer study to suggest that it is more important for FDA to gain a better understanding of how consumers use percent DV information rather than understand how consumers would use information on absolute amounts. The comment said that, according to its research, declaring absolute amounts on the label would decrease consumer attention to the percent DV information and would present ''significant implementation challenges.''

(Response) The comment refers to the study which we addressed in our response to comment 184. We are not aware of any evidence that including absolute amounts for the public health nutrients would detract from the percent DV information, and we intend to conduct consumer education on increasing the understanding of the percent DVs.

10. Single and Dual Column Labeling

The preamble to the proposed rule (79 FR 11879 at 11952 through 11953) noted that we have preexisting regulations for voluntary dual column labeling and that dual column labeling is mandatory for products that are promoted on the label, or in advertising, for a use that differs in quantity by twofold or greater from the use upon which the reference amount was based (*e.g.,* liquid cream substitutes promoted for use with breakfast cereals) (§ 101.9(b)(11)). The proposed rule would require (under certain conditions) dual column labeling where nutrition information would be presented based both on the serving size and on the entire package or unit of food.

We respond to comments on single and dual-column labeling in the final serving size rule.

(Comment 504 and Response) We address comments regarding dual column labeling in the final rule on ''Food Labeling: Serving Sizes of Foods That Can Reasonably Be Consumed At One Eating Occasion; Dual-Column Labeling; Updating, Modifying, and Establishing Certain Reference Amounts Customarily Consumed; Serving Size for Breath Mints; and Technical Amendments'' which is published elsewhere in this issue of the **Federal Register**.

11. The Footnote

Our preexisting regulations, at § 101.9(d)(9)(i), require the Nutrition Facts label to bear an asterisk after the ''% Daily Value'' declaration; the asterisk refers to a footnote that reads: ''*Percent Daily Values are based on a 2,000 calorie diet. Your Daily Values may be higher or lower depending on your calorie needs.'' Our preexisting regulations also require, below the footnote, a table that lists DRVs for total fat, saturated fat, cholesterol, sodium, total carbohydrate, and dietary fiber based on 2,000 and 2,500 calorie diets (§ 101.9(d)(9)(i)). However, the preamble to the proposed rule (79 FR 11879 at 11953) explained that the percent DV is not described in the footnote or anywhere else on the Nutrition Facts label, and so we wondered if such a description would help improve consumer understanding of the percent DV information. We also noted that consumers did not understand what was being conveyed in the footnote or the DRV table (id.). Consequently, we proposed to remove the requirement for the footnote table and to reserve a subparagraph (proposed § 101.9(d)(9)) for a future footnote. The preamble to the proposed rule (79 FR 11879 at 11953) also stated our tentative view that a new, simple footnote was needed to help consumers understand the meaning of the percent Daily Value. We said that the new footnote should have a larger type size, be more noticeable than the preexisting footnote, and include a statement that 2,000 calories a day is used for general nutrition advice (id.).

We also stated in the preamble of the proposed rule (id. at 11953 through 11954) that we would continue to conduct research during the rulemaking process to evaluate how variations in label format, including percent DV information in the footnote area, may affect consumer understanding and use of the Nutrition Facts label and that we would make the results of our study available for public review and comment.

In the preamble to the supplemental proposed rule (80 FR 44303 at 44306 and 44309), we described an experimental study on consumer responses to Nutrition Facts labels with various footnote formats. (We summarize the footnote study at part II.B.5.) The supplemental proposed rule would add language to the space reserved in proposed § 101.9(d)(9) to explain that the % Daily Value tells how much a nutrient in a serving of food contributes to a daily diet and that 2,000 calories a day is used for general nutrition advice. The supplemental proposed rule also would create an exemption to the proposed footnote requirement in § 101.9(d)(9) for the foods that can use the terms ''calorie free,'' ''free of calories,'' ''no calories,'' ''zero calories,'' ''without calories,'' ''trivial source of calories,'' ''negligible source of calories,'' or ''dietary insignificant source of calories'' on the Nutrition Facts label or in the labeling of foods as defined in § 101.60(b) because such products would have little to no impact on the average daily 2,000 calorie intake, which the footnote addresses. The supplemental proposed rule also would amend § 101.9(j)(13)(ii)(C) to allow the footnote to be omitted on small or intermediate-size packages (§ 101.9(j)(13)(ii)(A)(*1*) and § 101.9(j)(13)(ii)(A)(*2*)) provided that an abbreviated footnote statement (that % DV = % Daily Value) is used. Although the preamble to the supplemental proposed rule discussed allowing the footnote proposed in § 101.9(d)(9) to be omitted from products that qualify for a simplified format (§ 101.9(f)) (80 FR 44303 at 44309) provided that the abbreviated footnote statement is used, this provision was inadvertently omitted from the codified section of the supplemental proposed rule.

With respect to the Supplement Facts label, our preexisting regulations, at § 101.36(b)(2)(iii)(D), require that, if the percent DV is declared for total fat, saturated fat, total carbohydrate, dietary

fiber, or protein on the Supplement Facts label, a footnote state that ''Percent Daily Values are based on a 2,000 calorie diet.'' The proposed rule would require, for a product that is represented or purported to be for children 1 through 3 years of age and contains a percent DV declaration for total fat, total carbohydrate, dietary fiber, or protein, that a symbol be placed next to the percent DV declaration that refers the consumer to a statement at the bottom of the label that says ''Percent Daily Values are based on a 1,000 calorie diet'' (79 FR 11879 at 11947). We illustrated this footnote in a mockup of a Supplement Facts label depicting a multiple vitamin product for children and adults (§ 101.36(e)(11)(ii)). In the preamble to the proposed rule, we invited comments on whether changes to the footnote statement on the Supplement Facts label should be consistent with any changes that are made to the footnote statement in the Nutrition Facts label (79 FR 11879 at 11948). In the preamble to the supplemental proposed rule, we invited comments on whether we should replace the preexisting footnote in the Supplement Facts label with a footnote comparable to what we would require for the Nutrition Facts label; *i.e.,* ''2,000 calories a day is used for general nutrition advice'' (80 FR 44303 at 44307).

(Comment 505) Many comments supported removing the footnote table listing DRVs for certain nutrients based on 2,000 and 2,500 calorie diets. The comments said that the footnote table is confusing and difficult to read; consumers generally do not understand how to use it and probably derive little value from it; and the footnote occupies valuable label space that could be used for other information. However, other comments favored retaining the footnote table, indicating that it is useful for nutrition education purposes, may help consumers gain a perspective on their daily nutrient intake, and is a convenient reference for consumers who want this information.

Other comments suggested that the footnote should contain additional information beyond what is currently included or proposed. For example, some comments said the footnote should continue to explain that percent DVs are based on a 2,000 calorie diet and that an individual's Daily Values may be higher or lower depending on one's particular calorie needs. Some comments expressed concern that, without context, the public will not know whether 2,000 calories represents too many or too few calories. In addition, some comments said we should require language in the footnote explaining that growing children and adolescents may need more or less than 2,000 calories per day, depending on their age, gender, size, and activity level.

Other comments suggested that, because some consumers may view the label serving size as a recommended portion size, or use these terms interchangeably, we should include a footnote clarifying that ''serving size'' is based on the amount typically consumed and is not a recommended amount.

Another comment said that the Nutrition Facts label should go beyond just providing factual information and be a ''tool'' to help consumers make healthier food and beverage choices. For example, the comment said we should use a footnote to provide consumers with information about nutrients on the label that are ''beneficial'' (such as dietary fiber) or ''harmful'' (such as saturated fat) to their health. Several comments also said that we should consider including a link to a Web page where consumers can find more information about nutrition, health and calorie needs.

Several comments suggested that we seek a broader understanding of how consumers use the footnote. The comments emphasized that any revisions to the footnote should be based on research, and that the results of our consumer research should be made available to the public for review and comment. However, other comments would remove the footnote entirely, and some comments suggested that, as part of our consumer studies, we should evaluate whether a footnote is even needed. Several comments noted that the footnote itself is not an effective means for educating consumers and should not be used as an educational tool.

Several comments said that, regardless of which footnote was ultimately decided upon, the footnote should be succinct, occupy little space, and fit on small packages. Many comments emphasized that, because the proposed rule did not specify the exact footnote text and the amount of space the new footnote would require, it would be difficult to submit meaningful comments until further details were provided.

(Response) We agree with removing the footnote table listing DRVs for certain nutrients based on 2,000 and 2,500 calorie diets. As stated in the proposed rule (79 FR 11879 at 11953), we are aware of research suggesting that consumers do not understand what is being conveyed in the footnote table (Ref. 273). We also recognize that label space is limited and agree that eliminating the footnote table would free up space on the label that could be used for other purposes. Therefore, the final rule does not require the footnote table which lists the DRVs for total fat, saturated fat, cholesterol, sodium, total carbohydrate, and dietary fiber for 2,000 and 2,500 calorie diets.

We disagree with comments suggesting that a footnote be used to explain that calorie needs vary among population groups (including children and adolescents) or to clarify the meaning of ''serving size.'' The footnote area of the label is not an appropriate place for providing this information because of limited space on the label. Furthermore, we do not agree that it would be appropriate to use a footnote to indicate ''beneficial'' or ''harmful'' nutrients that are declared on the label, as the comment suggested. We considered a similar concept in the alternative visual format that was discussed in the preamble to the proposed rule (79 FR 11879 at 11995), but, after reviewing the comments on the proposed rule, indicated that we did not intend to consider the alternative format for the Nutrition Facts label further (see 80 FR 44302).

With respect to comments suggesting that we base revisions of the footnote (including the option of not having any footnote at all) on research and that our research results should be made available to the public for review and comment, we did conduct research on various footnote options and made those results publicly available (see 80 FR 44302; 80 FR 44303).

Finally, we do not agree with the comments stating that we should consider including a link to a Web page where consumers can find more information about nutrition, health and calorie needs. Information on the Nutrition Facts label should be available to the consumer at the time of product purchase or consumption.

(Comment 506) Many comments to the supplemental proposed rule supported FDA's proposed footnote, ''*The percent DV tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice,'' and generally agreed that the footnote should include both a definition of percent DV as well as a reference calorie level. The comments said that the proposed footnote conveys the information that consumers need to understand the significance of the percent DV declaration in the context of a daily diet and highlights factors (*i.e.,* nutrient values and total calorie intake)

that are important in making dietary decisions. Several comments also pointed out that, because the footnote has been condensed (*i.e.,* by removing the footnote table), it would help counterbalance the increased space requirements of the Nutrition Facts label.

Other comments objected to the proposed footnote and suggested alternative footnote text. For example, one comment said that the first sentence in the footnote is confusing grammatically; the second sentence does not flow naturally from the first sentence; it is unclear how the two concepts expressed in the footnote are related; and the proposed footnote text is longer than that of the current footnote and will take up too much valuable label space. The comment suggested an alternative footnote, ''*The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a 2,000 calorie daily diet.'' The comment said its suggested footnote is more concise and easier to follow.

Another comment said that the footnote should specify that a 2,000 calorie daily diet pertains to adults and suggested the following footnote text: ''The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice for adults.'' Another comment that criticized the proposed footnote for being ''too verbose'' and provided six different, but similar, versions of a ''more succinct'' alternative footnote, with one option reading as: ''* %DV = %Daily Value, how much a nutrient in a serving contributes to a daily 2,000 calorie diet.''

Several other comments either suggested modifications to the proposed footnote (*e.g.,* expanding the term ''food'' to ''food or beverage'' to emphasize that beverages also contribute to one's daily nutrient intake) or opposed the footnote because, according to the comments, the footnote was not tested and was not supported by research. Furthermore, several comments said that, because no significant differences were found among the footnotes in our consumer study, we should give further consideration to some footnotes that were tested, but ultimately rejected. In particular, the comments said we should reconsider the footnote which included the statement, ''5% or less is a little, 20% or more is a lot'' after the % Daily Value description (experimental footnote 2). The comments said that this guideline for what constitutes a ''lot'' or a ''little'' of a nutrient may be helpful to consumers in judging the nutrient content of a particular product. One comment also expressed support for the footnote stating, ''These are nutrients to reduce in your diet,'' with the footnote symbol inserted to the left of the listings for saturated fat, *trans* fat, cholesterol, sodium, and sugars in the Nutrition Facts label (experimental footnote 5). The comment said that this footnote scored well in our consumer study and offers ''real value'' for consumers seeking information on nutrients in the diet that should be reduced.

(Response) We appreciate the suggestions for modifying or refining the footnote. However, the alternative footnote statements do not offer a significant improvement over the footnote text that we have proposed. Furthermore, the comments did not provide any evidence or data indicating that any alternative footnote represented an improvement over the proposed footnote.

The second statement of our proposed footnote, ''2,000 calories a day is used for general nutrition advice,'' is the same as the succinct statement that will be required on menus and menu boards under FDA's menu labeling final rule (79 FR 71156 (December 1, 2014)). Moreover, by including this statement as a separate, stand-alone sentence in the footnote text, we provide consistency between labels on packaged foods and those on foods sold in restaurants. Adding the words ''for adults'' at the end of this sentence, as one comment suggested, would undermine this consistency, take up additional space, and is not needed because the Nutrition Facts label is intended to apply to individuals 4 years of age and older (with the exception of labels on products other than infant formula represented or purported to be specifically for infants through 12 months of age and children 1 through 3 years of age). Furthermore, as we explain in part II.E.3, a 2,000 calorie reference intake level is applicable to the general population and is used as the basis for setting DRVs for total fat, saturated fat, total carbohydrate, dietary fiber, and protein, so there is no need to add the words ''for adults'' in the footnote text.

Regarding the comment suggesting the modified footnote text, ''The % Daily Value (DV) tells you how much a nutrient in a serving of food contributes to a 2,000 calorie daily diet,'' the statement is brief and grammatically correct, but may not be technically correct because the daily values of some declared nutrients, such as sodium and cholesterol, do not depend on the caloric intake. Therefore, it would not be accurate to link the percent DV in a serving ''to a 2,000 calorie daily diet,'' as stated in the modified footnote, rather than ''to a daily diet'' as stated in our footnote.

Although we agree that including ''5% or less is a little, 20% or more is a lot'' after the % Daily Value description (experimental footnote 2) can be helpful in judging the nutrient content of a particular product, we note that our consumer research study did not demonstrate that this footnote performed any better than the other footnotes that we investigated. As we explained in the preamble to the supplemental proposed rule (80 FR 44303 at 44306), our results indicated that none of the modified footnotes we tested significantly affected consumer perceptions of the products or judgments of nutrient levels; all five footnote options elicited similar perceptions and judgments relative to the current footnote and a no-footnote control. We also are concerned that including this qualifying phrase would increase the amount of space required for the footnote. However, as we stated in the preamble to the proposed rule (79 FR 11879 at 11954), the ''5/20 rule'' can be used as a general frame of reference for evaluating the nutrient content of foods. We anticipate that explaining this approach for using the percent DV information will be a part of our future consumer education efforts, so it would not be necessary to include an explanation of the ''5/20 rule'' in the footnote.

As for the comments that favored consideration of the footnote which indicated ''nutrients to reduce in your diet'' (footnote 5), we previously considered this concept in our ''alternative format'' (79 FR 11879 at 11995), but found it offered no clear advantages over the current and proposed formats in helping consumers to identify specific information on the label or to make healthier food choices.

We do not agree with the comment that said our proposed footnote is ''confusing grammatically.'' We deliberately used language that was informal rather than grammatically rigid or technical. Our intent was to make the footnote consumer friendly. We also consider our footnote to be simple and brief in providing a description of the percent Daily Value, which is lacking in the preexisting footnote.

Finally, we decline to include the word ''beverage'' in the footnote. The term ''food'' is defined in section 201(f)(1) of the FD&C Act as including articles used for both ''food or drink.'' Moreover, the Nutrition Facts label has

appeared on beverages for more than 20 years, so consumers should understand that the entire label, including the footnote, applies to foods that are beverages.

We expect that our footnote, which explains the term ''% Daily Value'' and provides a reference calorie level, will assist consumers in better understanding the information on the Nutrition Facts label and in maintaining healthy dietary practices. Therefore, the final rule, at § 101.9(d)(9), requires a footnote stating that, ''* The % Daily Value tells you how much a nutrient in a serving of food contributes to a daily diet. 2,000 calories a day is used for general nutrition advice,'' in all Nutrition Facts label formats except for the exemptions previously noted. The final rule also requires, on labels of products represented or purported to be for children 1 through 3 years of age, that the second sentence of the footnote substitute ''1,000 calories'' for ''2,000 calories,'' so the footnote statement will read: ''* The % Daily Value tells you how much a nutrient in a serving of food contributes to a daily diet. 1,000 calories a day is used for general nutrition advice.''

(Comment 507) Many comments supported the exemption for a footnote on products containing a negligible amount of calories and that can use the term ''calorie free'' or one of its synonyms. The comments agreed that a footnote which addresses a 2,000 calorie intake is not relevant for these products, and the exemption would be a practical way of conserving label space for the nutrient declarations that are required.

However, other comments opposed the exemptions because, according to comments, products that have little or no impact on calorie intake still may contain substantial amounts of nutrients such as vitamins and minerals. As an example, one comment said that fortified beverages may contain significant amounts of electrolytes as well as 100 percent of the DV of certain vitamins. The comment suggested that ''calorie free'' products include the first sentence of the footnote, ''The % Daily Value tells you how much a nutrient in a serving of food contributes to a daily diet'' because it would help consumers understand the vitamin and mineral content of these calorie-free foods.

Other comments supported the use of an abbreviated footnote, such as ''% DV = % Daily Value'' on the simplified format label and on labels of small and intermediate-size packages. Some comments explained that an abbreviated footnote would save label space. However, one comment opposed allowing the abbreviated footnote to be used on small and intermediate-size packages because, according to the comment, such products are often high in added sugars and are routinely marketed to children and adolescents. The comment suggested that consumers would benefit by having the complete footnote appear on these food packages.

(Response) As we explained in the preamble to the supplemental proposed rule (80 FR 44303 at 44309), we are applying the same rationale in this final rule that we used in the 1993 final rule with regards to exempting small and intermediate-size packages from some of the footnote language we required for larger products. The 1993 final rule gave manufacturers flexibility in using the complete footnote on all product labels. We recognized that the benefits of requiring this footnote were not relative to the specific product that carries the information and that the information would be available to consumers if it appeared on a significant percentage of food labels (58 FR 2079 at 2129). Therefore, although the final rule does not require any footnote on these products, we will allow the voluntary use of the first part of the footnote statement, ''* The % Daily Value tells you how much a nutrient in a serving of food contributes to a daily diet'' on products that can use the terms ''calorie free,'' ''free of calories,'' ''without calories,'' ''trivial source of calories,'' ''negligible source of calories,'' or ''dietary insignificant source of calories'' on the label or in the labeling of foods, as defined in § 101.60(b).

We acknowledge that small and intermediate-size packages may be high in added sugars and marketed to children and adolescents. However, both the absolute amount and % DV of added sugars will be declared on labels of small packages, so this information will be available to consumers. We also recognize the need to conserve space on smaller packages, which is why we allow other adjustments, such as not requiring the declaration of absolute amounts of the public health nutrients and the use of the tabular (§ 101.9(j)(13)(ii)(A)(*1*)) and linear (§ 101.9(j)(13)(ii)(A)(*2*)) display on small packages and intermediate-size packages having a total surface area available to bear labeling of 40 or less square inches. Therefore, the final rule does not require the footnote in § 101.9(d)(9) to be used on products in small packages as specified in § 101.9(j)(13)(ii)(A)(*1*) and § 101.9(j)(13)(ii)(A)(*2*), but manufacturers may voluntarily include the abbreviated footnote ''% DV = % Daily Value'' on these packages and in a type size no smaller than 6 point. Furthermore, the final rule does not require the footnote in § 101.9(d)(9) to be used on products that qualify for using the simplified format, as explained in § 101.9(f)(5), provided that the abbreviated footnote ''% DV = % Daily Value'' in a type size no smaller than 6 point is used on these package labels when Daily Value is not spelled out in the column heading.

Finally, in the preamble to the proposed rule (79 FR 11879 at 11953), we recognized that the footnote, by appearing in a small type size at the bottom of the label, may be less noticeable to consumers and of less use than if it had been larger and otherwise more noticeable. Consequently, our tentative view was that increasing the type size of the footnote would assist consumers in using the information, and we requested comments on this issue. We did not receive any comments that supported increasing the type size of the footnote (although comments supported increasing the font size for certain other declarations, *e.g.,* ''Calories'' and ''Serving size''), but some comments supported using as little space as possible for the footnote information. Therefore, the final rule does not affect the pre-existing requirement in § 101.9(d)(1)(iii) that specifies that the information required in § 101.9(d)(9) be in a type size no smaller than 6 point.

(Comment 508) Many comments discussed whether there should be a footnote on the labels of foods represented for infants 7 to 12 months of age or children 1 through 3 years of age. Most comments supported having a footnote on the label of foods intended for these subpopulation groups. For example, one comment said that a voluntary footnote should be permitted for foods specifically marketed to children 1 through 3 years of age and that the footnote should state, ''Percent Daily Values are based on a 1,000 calorie diet.'' Other comments said that both conventional foods and dietary supplement products marketed for these age groups should have a footnote (denoted by an asterisk) indicating the number of calories that the percent DVs listed on the labels is based on. One comment noted that this had already been proposed for dietary supplements (79 FR 11879 at 11947). The comment further suggested that information about percent DVs of nutrients for different age groups be made available online (arranged by age group) so that parents and others interested in nutrition would have ready access to this information.

Another comment suggested that we allow a voluntary footnote stating ''Total fat and cholesterol should not be limited in the diets of children less than 2 years

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unless directed by a physician'' to provide dietary guidance to parents and other caregivers to help assure total fat is not restricted in the diet of young children. The comment said that the American Academy of Pediatrics recommends not restricting fat or cholesterol for infants and children younger than 2 years of age, as rapid growth and development occur during this time, necessitating a high energy intake. Another comment said we should not finalize the rule until we had conducted appropriate research, including consumer testing, to better understand the impacts of declaring saturated fat and cholesterol on the labels of products represented or purported to be specifically for infants and children 1 through 3 years of age and if an explanatory footnote would assist in improving consumer understanding when accompanying any relative declaration.

(Response) We recognize that the percent DVs of certain nutrients (*e.g.,* fats, carbohydrates, protein) for foods specifically intended for children 1 through 3 years of age are based on a reference calorie intake of 1,000 calories/day. However, as explained in part II.O (Subpopulations), the IOM's quantitative intake recommendations (AIs and RDAs), rather than a calorie level, provide a basis on which to determine RDIs (and percent DVs) for vitamins and minerals for this subpopulation. Although the comments suggested including the footnote ''Percent Daily Values are based on a 1,000 calorie diet'' on labels of foods specifically intended for children 1 through 3 years of age, this statement would not be accurate for all nutrients. Therefore, as illustrated in the label mockup in § 101.9(j)(5)(ii), the final rule requires the labels of these food products to have a footnote that includes the statement ''1,000 calories a day is used for general nutrition advice;'' this information would parallel the footnote statement used on food labels for the general population (*i.e.,* 4 years of age and older).

With respect to the comment suggesting we allow a voluntary footnote stating that total fat should not be limited in the diets of children less than 2 years unless directed by a physician (or similar wording), we acknowledge, in general, that total fat should not be limited in the diets of young children less than 2 years of age unless directed by a health professional (as previously explained in part II.O, Subpopulations). Because the final rule requires the mandatory declaration of saturated fat and cholesterol on labeling for infants and children, we are continuing to consider how a voluntary footnote explaining that total fat should not be restricted in the diets of children less than 2 years of age may help caregivers maintain healthy dietary practices for these subgroups, and how the information can be conveyed effectively. Although, for this final rule, we decline to allow this voluntary statement to be located within the Nutrition Facts label, manufacturers may place this or a similar statement in another area of the package, provided the statement is truthful and not misleading. We intend to engage in education efforts to explain changes to the Nutrition Facts label and will include labeling of foods for infants and children 1 through 3 years of age in these efforts.

(Comment 509) One comment said that the Supplement Facts label should be similar to the Nutrition Facts label used for conventional foods because different versions of the labels may decrease consumer use, understanding and trust. However, it was not clear if the comment was referring specifically to the footnotes of these labels. Another comment said there should not be a footnote on the Supplement Facts labels because consumers do not receive nutrition solely from these products, so a footnote referring to total calories would be unnecessary. The comment added that, because nutrition calculations are based on 2,000 calories, this information is already standardized across the industry, making the notation unnecessary.

Another comment expressed concern that the statement ''2,000 calories a day is used for general nutrition advice'' on Supplement Facts labels would not be useful to consumers in the absence of additional information. However, the comment said it would be difficult to include additional, explanatory text because of limited space, especially on small packages. Therefore, the comment would retain the preexisting footnote, ''Percent Daily Values are based on a 2,000 calorie diet,'' on Supplement Facts labels.

(Response) We agree that information about calories is not relevant for many dietary supplement products because the products contain only vitamins and minerals and do not contain nutrients that provide calories, such as total fat, saturated fat, total carbohydrate, and protein. Therefore, the footnote in previously required § 101.9(d)(9) would not be appropriate on Supplement Facts labels for products that do not contain these calorie sources. Furthermore, dietary supplements are intended to supplement the diet, and the information in the footnote for conventional foods that references 2,000 calories as a basis for ''general nutrition advice,'' or explains percent DV in the context of what a serving contributes to a daily diet, is for a different use from that of dietary supplements.

Although the intent of the comment regarding the need for consistency between the Nutrition Facts label and Supplement Facts label is not clear, we recognize the necessity of having different footnotes on labels of conventional foods and dietary supplements, consistent with how these products are used. Therefore, the final rule retains the preexisting footnote on Supplement Facts labels and amends the list of macronutrients, for when the footnote is required, to include added sugars. Therefore, the final rule requires a footnote if the percent of Daily Value is declared for total fat, saturated fat, total carbohydrate, dietary fiber, protein, or added sugars), stating that ''Percent Daily Values are based on a 2,000 calorie diet'' (§ 101.36(b)(2)(iii)(D)) because that information is related to the calorie contribution of the calorie-containing ingredients. The footnote statement for Supplement Facts labels does not contain the statement required for conventional foods that states ''The % Daily Value tells you how much a nutrient in a serving of food contributes to a daily diet.'' In addition, if a product declares a percent DV for total fat, saturated fat, total carbohydrate, dietary fiber, protein, or added sugars, and is represented or purported to be for use by children 1 through 3 years of age, the final rule, at § 101.36(b)(2)(iii)(D), requires a footnote statement, ''Percent Daily Values are based on a 1,000 calorie diet.''

(Comment 510) One comment asked us to clarify the footnote's width because the width requirements were not specified. The comment said that this issue would be particularly important when either the tabular format (§ 101.9(d)(11)(iii)) or the dual column tabular format (§ 101.9(e)(6)(ii)) was used because, without a specific width requirement, the footnote text could be wrapped in various ways, resulting in the footnote occupying space varying from being mostly horizontal (*i.e.,* wide and short) to mostly vertical (*i.e.,* narrow and tall). The comment suggested the possibility of specifying a minimum width that would require at least the words ''The % Daily Value'' to fit on a single line.

(Response) Manufacturers have the flexibility, within certain parameters, in how they display the footnote to satisfy the configuration and design constraints of their packages. Therefore, we decline to specify a minimum number of words

per line for the footnote, as the comment suggested. However, we intend to monitor how firms comply with the format requirements, including the footnote display. If we determine that manufacturers are having difficulty fitting the footnote text and other required information within the Nutrition Facts label, we will consider whether further action, including rulemaking, is needed with regard to positioning the footnote.

12. Use of Highlighting With a Type Intermediate Between Bold or Extra Bold and Regular Type

Under our preexisting regulations, only nutrients that are not indented (*i.e.,* ''Calories,'' ''Total Fat,'' ''Cholesterol,'' ''Sodium,'' ''Total Carbohydrate,'' and ''Protein'') on the Nutrition Facts label are required to be highlighted in bold or extra bold type or other highlighting (§ 101.9(d)(1)(iv)). In the preamble to the proposed rule (79 FR 11879 at 11954), we stated that, based on design considerations of using bold type to help differentiate the name of the nutrient from its absolute amount (Ref. 262), all of the other nutrients listed on the Nutrition Facts label, including those that are indented and the vitamins and minerals, should also be highlighted to help set the names of the nutrients apart from other information that appears on the label. The key nutrients that are not indented would still be highlighted in a font that is bolder than the indented nutrients, so the overall style of the Nutrition Facts label would not change. Thus, we proposed to amend § 101.9(d)(1)(iv) to remove the restriction that prohibits any other information on the label to be highlighted and to require that all voluntary nutrients specified in § 101.9(c), including the vitamins and minerals listed in § 101.9(c)(8)(iv), appear in a type intermediate between bold and regular type (if bold type is used) or between extra bold and regular type (if extra bold type is used) on the Nutrition Facts label.

(Comment 511) One comment suggested that if too much information on the Nutrition Facts label was bolded, nothing would stand out. The comment also said that too much bolding would be especially problematic for small packages because it would be difficult to maintain legibility of the printed information. The comment said that small print that is bolded would be even more difficult to read, because the letters would appear to run together even more.

Another comment suggested that, as an alternative to bolding, we might want to reconsider the restriction of using reverse highlighting (*i.e.,* white text printed in a black box, also known as reverse printing) as a method of increasing prominence. The comment stated that since the Nutrition Facts label was introduced in 1993, vast improvements have been made in printing technologies and capabilities, which should help alleviate previous concerns with regards to whether reverse printing could meet minimum printing tolerances.

(Response) We agree that too much bolding may reduce the contrast between information that is intended to be relatively more or less prominent on the Nutrition Facts label and that maintaining adequate resolution of printed information on labels of small packages might be particularly difficult. We also agree that it is more likely that letters or numbers may run together when information is highlighted, especially on labels of small packages, and we note that our preexisting regulations (§ 101.9(d)(1)(ii)(D)) specify that letters on the Nutrition Facts label should never touch. Therefore, based on the graphic design principle of using contrast to distinguish differences between adjacent items that would otherwise appear similar, and the importance of preserving adequate resolution to ensure the sharpness and clarity of the label information, the final rule does not amend the portion of proposed § 101.9(d)(1)(iv) that would require the indented nutrients and the vitamins and minerals (except sodium) to be highlighted in a type intermediate between bold or extra bold type and regular type.

As for the comment suggesting that we reconsider the use of reverse printing, we had concluded in the 1993 final rule (58 FR 2079 at 2137), based on comments and the professional literature at that time, that the use of reverse printing on the Nutrition Facts label would give rise to technical and legibility problems, especially on small containers, and therefore we declined to permit reverse printing as a form of highlighting (§ 101.9(d)(1)(iv)). While advances in technology may have removed some previous barriers that existed with this printing technique, we need to learn more about the technology before we consider revising the rule to address reverse printing.

13. Addition of a Horizontal Line Beneath the Nutrition Facts Heading

Our preexisting regulations, at § 101.9(d)(2), require that the Nutrition Facts heading be set in a type size larger than all other print size in the nutrition label (§ 101.9(d)(2)) but does not require that this heading be set apart from the rest of the label with a horizontal hairline rule, which is a thin line. Horizontal lines are used throughout the Nutrition Facts label as a key graphic element to divide space, direct the eye, and give the label a unique and identifiable look. The proposed rule would require that a thin horizontal line (*i.e.,* a 0.25 point hairline rule) be inserted directly beneath the Nutrition Facts heading with the exception of the linear display for smaller packages in § 101.9(j)(13)(ii)(A)(*2*).

(Comment 512) One comment said that the hairline rule beneath the Nutrition Facts title improves the overall appearance of the Nutrition Facts label and its ''ease of use.'' Another comment said that the use of horizontal lines and other design elements (*e.g.,* white space, bold fonts, etc.) are visual cues that draw attention to important information on the Nutrition Facts label, helping to improve readability and make the information easier to process and remember. Another comment said that a horizontal line beneath the Nutrition Facts heading would help separate the heading from the ''____ servings per container'' declaration, because all of the information in the first two lines of the label was presented in bold type.

(Response) We agree that a thin horizontal line directly beneath the Nutrition Facts heading would make the heading more visually appealing. Our requirement in § 101.9(d)(1)(v) to insert the horizontal line beneath the Nutrition Facts heading for all formats (except the linear display for smaller packages described in § 101.9(j)(13)(ii)(A)(*2*)) is based on graphic design principles and other design considerations previously discussed in the preamble to the proposed rule.

14. Replacing ''Total Carbohydrate'' With ''Total Carbs''

Nutrition information declared on the Nutrition Facts label must be presented using the nutrient names specified in § 101.9(c) or § 101.9(j)(13)(ii)(B). According to § 101.9(c)(6), the nutrient name used for listing information about the carbohydrate content of a product is ''Total Carbohydrate.'' Certain abbreviations, as specified in § 101.9(j)(13)(ii)(B), may be used on the Nutrition Facts label on packages that have a total surface area available to bear labeling of 40 or less square inches.

In the preamble to the proposed rule (79 FR 11879 at 11954), we explained that replacing ''Total Carbohydrate,'' the nutrient name currently required on most formats, with the shorter term ''Total Carbs'' would maximize white space, maintain simplicity, and because

it is a commonly used term, help the public to readily observe and comprehend the nutrition information presented in the Nutrition Facts label.

(Comment 513) Most comments objected to replacing ''Total Carbohydrate'' with ''Total Carbs'' on the Nutrition Facts label. Several comments referred to the term ''Total Carbs'' as being ''jargon,'' ''slang,'' ''sloppy,'' or ''denigrating.'' Other comments stated that ''Total Carbohydrate'' is a term that is familiar to consumers, is frequently used in the media, and has appeared on the Nutrition Facts label for more than 20 years. The comments also noted that ''carbohydrate'' is the correct, scientifically accurate term specified in the FD&C Act and NLEA and is used in the DGA, IOM reports, and other government or scientific documents.

One comment questioned whether any data exist suggesting that consumers are either confused by the word ''carbohydrate'' or would understand the term ''carbs'' any better. Another comment suggested that research is needed to evaluate whether the proposed change would affect consumer use and understanding of the carbohydrate information presented on the label.

Many comments said that listing the total carbohydrate content in a serving of food as ''Total Carbs'' rather than ''Total Carbohydrate'' could have a negative impact on the ability of people with diabetes to accurately assess their carbohydrate intake and thus their ability to manage their disease. The comments explained that diabetics, who monitor their blood glucose levels and adjust their insulin requirements accordingly, must be able to accurately determine the carbohydrate content of their foods, such as through ''carbohydrate counting.'' Several comments pointed out that many diabetics, especially those who are newly diagnosed, recognize the term ''carb choice'' or ''carb serving'' as referring to a serving of food that contains 15 grams of total carbohydrate. The comments noted that, in this context, the word ''carb'' has a specific meaning, and that declaring ''Total Carbs'' on the Nutrition Facts label could cause confusion and result in diabetics taking the wrong dose of insulin.

Other comments suggested that ''carb'' or ''carbs'' frequently carries a negative connotation when it is linked to a ''low carb'' diet, the ''net carbs'' of a product, or to ''carb loading'' before an athletic competition. The comments expressed concerns that the term may be used in a context that does not support healthy dietary practices. One comment noted that the term ''carbs,'' if perceived negatively, could inadvertently challenge advice to consume 65 percent of calories from carbohydrates, as recommended in the 2010 DGA. Another comment questioned why carbohydrates should be treated differently than other nutrients on the Nutrition Facts label because it would be the only abbreviated nutrient on most label formats.

One comment said that, because previous research suggests that consumers have difficulty understanding acronyms and abbreviations, the term ''carbs'' may not be appropriate on the label, and may present an additional challenge on bilingual labels. Another comment indicated that if the final rule uses ''Total Carbs,'' the ''Added Sugars'' declaration would become more prominent, leading to consumer confusion and distracting from an overall focus of reducing calorie consumption from all macronutrient sources.

Some comments supported replacing the term ''Total Carbohydrate'' with ''Total Carbs'' and said that ''carbs'' is a term that is part of the daily vocabulary of many people and the term would ''draw their attention'' which could be beneficial.

(Response) We acknowledge that ''carbohydrate'' is the correct, scientifically accurate term used in government or scientific documents and that ''carbs'' may be perceived as jargon. We further recognize the possibility that some diabetics may have difficulty distinguishing between the terms ''Total Carbs,'' ''carb choice,'' and ''carb serving,'' but note that the Nutrition Facts label, and any associated changes in format resulting from this rulemaking, applies to the general healthy population rather than to those with a specific disease. We are unaware of any data suggesting that consumers would be confused by the abbreviation ''Carbs'' or that this term would adversely affect the ability of consumers to interpret other parts of the Nutrition Facts label, or adversely impact dietary advice, as suggested by some comments. Furthermore, we already permit the abbreviation ''carb.'' (singular) for ''carbohydrate'' on small packages having space constraints, as specified in § 101.9(j)(13)(ii)(B), and we note that the term ''carbohydrate'' is spelled out on the Nutrition Facts label of most food products and therefore is readily observable for consumers who might be confused by the abbreviated term on small packages. However, because ''carbs'' (plural) may be perceived as an informal term and may have a negative connotation for some individuals and because a ''Total Carbs'' declaration may be problematic on some bilingual labels when this term is used instead of ''Total Carbohydrate'' generally, we will continue to require that ''Total Carbohydrate'' be used as the nutrient name for carbohydrates, as specified in § 101.9(c)(6), and that ''Total carb.'' continue to be the abbreviation for this term (*e.g.,* as applicable on small packages) as specified in § 101.9(j)(13)(ii)(B).

15. Alternative Visual Formats/Fonts

We did not propose any changes to the basic format of the Nutrition Facts label, as specified in § 101.9(d)(12), because we were unaware of any evidence that would support an alternative format. However, the preamble to the proposed rule did contain a mockup of an alternative concept for the Nutrition Facts label format (79 FR 11879 at 11955) that categorized nutrient declarations as ''quick facts'' about certain nutrients, nutrients to ''avoid too much'' of, and nutrients to ''get enough of,'' and we invited comment on whether we should require a specific type style for the Nutrition Facts label.

After reviewing the comments on the proposed rule, we tentatively concluded that we did not intend to further consider the alternative format for the Nutrition Facts label (80 FR 44302). Most comments agreed with our tentative conclusion, and other comments raised questions that we may consider if we decide to conduct further research on this issue in the future. A review of the results of FDA's consumer research, which we made available in reopening of the comment period as to specific documents (80 FR 44302), did not provide information to change our tentative conclusion, so we are not giving further consideration to the alternative format as part of this rulemaking.

16. Miscellaneous Comments

a. *Size and space issues.* The preamble to the proposed rule did not invite comments on whether our proposed format changes would affect the ability of small packages to accommodate the Nutrition Facts label. Our intention was to use graphic design principles to improve the overall visual appearance of the Nutrition Facts label formats without altering the labels' dimensions. However, several comments addressed this issue, particularly with regards to the use of the proposed linear format on small and very small food packages.

(Comment 514) Many comments said the proposed Nutrition Facts label formats appeared to be larger than the preexisting label formats and, therefore, would take up too much space on food packages. The comments said that implementing many proposed changes, such as increasing the prominence of ''servings per container and the ''calorie'' information as well as adding a line for ''Added Sugars,'' would necessarily increase label size. One comment suggested that we did not adequately consider how the proposed Nutrition Facts labels would fit on actual food products and asked us to ''verify'' that the proposed formats would not result in larger labels. Several comments said that companies would need to redesign their packages to accommodate the increased amount of space that would be necessary for labels to comply with the proposed format changes and to fit on packages, resulting in significant costs to the industry.

Other comments indicated that, for all of the required information to fit within the boundaries of certain proposed formats, some labels would be cluttered, difficult to read, and challenging for consumers to use. One comment said that the label's overall visual appearance would be dense, complex, cluttered, and contradict FDA's intent to maintain the NLEA requirements. The comment said that the Nutrition Facts label should have a simple format, minimize clutter, and enable consumers to observe and comprehend the information readily.

Several comments emphasized that a larger nutrition label would occupy ''valuable'' package space that could be used for other purposes. One comment said that a larger Nutrition Facts label might reduce the available package space that could be used for marketing and promotional messages, and this would be of particular concern to small firms unable to afford advertising costs. Another comment said that the proposed format changes might limit the amount of space on packages that could be used for product recipes and cooking instructions (*e.g.,* information about proper cooking times and temperature settings) which may be necessary for ensuring food safety.

(Response) We disagree with the comments suggesting that the proposed formats would be significantly larger than the current formats. Each label was specifically designed to occupy the same amount of package space as the preexisting label. While some nutrient information will be declared in a larger font size and style compared to the preexisting format, and the final rule requires the declaration of ''Added Sugars'' information, we are also removing the requirement for the ''Calories from Fat'' declaration and reducing the amount of space that will be necessary for the footnote. In certain cases (*e.g.,* on labels of foods represented or purported to be specifically for infants through 12 months of age or on labels of foods that can use the terms ''calorie free,'' ''free of calories,'' ''no calories,'' ''zero calories,'' ''without calories,'' ''trivial source of calories,'' ''negligible source of calories,'' or ''dietary insignificant source of calories'' on the Nutrition Facts label or in the labeling of foods as defined in § 101.60(b)), we are removing the footnote requirement altogether. We also note that we are reducing the type size of the numerical value for calories, from 24 point to 22 point, and 14 point for the tabular display and linear display for smaller packages with a total surface area available to bear labeling of 40 square inches or less in § 101.9(j)(13)(ii)(A)(*1*) and (*2*). Taken together, these format modifications will not result in a significant change in the size of the labels. Therefore, we decline to ''verify'' that the revised formats will not be larger than the current ones and disagree that manufacturers will need to redesign packages extensively to accommodate the revised Nutrition Facts labels. Also, because we are not requiring that absolute amounts be listed for voluntary nutrients, we do not anticipate that excessive crowding will be problematic on labels with multiple columns, such as those on breakfast cereal packages which list nutrition information for the product as packaged, as served (*e.g.,* with milk), and for a subpopulation (*e.g.,* children less than 4 years of age). Although providing nutrition information for these categories is voluntary, if a manufacturer chooses to use such multiple columns and adequate space is not available on the side panel, the Nutrition Facts label may be placed on the back panel of the package (as provided for in § 101.2(a)(1)) where more space is likely to be available.

With respect to the comment regarding the need for small businesses to have adequate space on packages for promotional and marketing messages, we acknowledge the importance of communicating information about the product. Similarly, we recognize the importance of providing consumers with information about food preparation, recipes, and safety issues relative to the product. However, as specified in § 101.9(j)(17), non-mandatory label information on the package information panel (as described in § 101.2(a)) is not considered to be a factor in determining the sufficiency of available space for the placement of the Nutrition Facts label. Therefore, all manufacturers, regardless of size, who are required to display the Nutrition Facts label on its products must follow the regulations with regards to general food labeling requirements and provisions as discussed in § 101.1 through 101.5.

(Comment 515) Several comments noted that label space, which is already limited, would be further constrained on bilingual labels. The comments suggested that bilingual labels will become increasingly common and that we should provide examples of bilingual labels for further public comment.

(Response) The use of bilingual Nutrition Facts labels is voluntary. We do not agree that our format changes will prevent manufacturers from using a bilingual label, as many options are available regarding where the label is located on a package (*e.g.,* the back panel). We have provided an example of a bilingual Nutrition Facts label in ''A Food Labeling Guide: Guidance for Industry'' (Ref. 122). Manufacturers who use a bilingual label can review this guidance document. We anticipate that future updates will be made to ''A Food Labeling Guide: Guidance for Industry'' to correspond to format changes in the final rule.

(Comment 516) One comment said that, because the standard format requires both percent DV and absolute amounts of mandatory vitamins and minerals to be declared, there would not be enough space on some packages to allow the nutrients of public health concern to be listed side by side in two columns (as specified in § 101.9(d)(8)), which the comment called a ''space saving feature.'' The comment provided an example of a label demonstrating that it is not possible to list micronutrients in two columns because of layout constraints caused by the package's configuration. The comment said that although the proposed Nutrition Facts label changes were intended to have a minimum impact on product packages, layout constraints in some cases would necessitate significant package redesign to comply with the revised format. The comment suggested that we had not adequately considered certain package shapes where changes in format would have ''consequential'' effects on package design.

(Response) We acknowledge there are layout constraints with certain packages, but we have given manufacturers flexibility in how they apply the Nutrition Facts label on

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products having significant size and space challenges. The comment's example used certain text sizes and bolding that were initially proposed, but are not included in the final rule, so the comment's example, under the final rule's requirements, would take up less space. In response to concerns of products that have significant size and space constraints we are removing the requirement for the footnote statements in § 101.9(j)(13)(ii)(C) for the tabular format for small packages as shown in § 101.9(j)(13)(ii)(A)(*1*) and the linear format as shown in § 101.9(j)(13)(ii)(A)(*2*), however, the abbreviated footnote ''% DV = % Daily Value'' may be used on these packages. Because we are removing the requirement in § 101.9(j)(13)(ii)(C), we are redesignating § 101.9(j)(13)(ii)(D) as § 101.9(j)(13)(ii)(C). We also are allowing ''vitamin'' to be abbreviated as ''vit.'' and potassium to be abbreviated as ''Potas.'' in § 101.9(j)(13)(ii)(B) which will further conserve space. Although we cannot predict all the different sizes and shapes of packages that may enter the marketplace, we permit various formats of the Nutrition Facts label and allow flexibility in order to accommodate packages having various design features.

(Comment 517) Many comments said that the proposed linear display for small packages (illustrated in § 101.9(j)(13)(ii)(A)(*2*) (79 FR 11879 at 11979)) would not fit on many small packages, such as those for candy, chewing gum, and other confectionery products, because it occupies substantially more space than the current linear display format. Some comments included detailed mockups of complete small product packages demonstrating that, due to their shape or size, some packages would not be able to accommodate the proposed Nutrition Facts labels without obscuring some information on the package or label, even if a minimum legible font size of 6 point was used on the label. Other comments pointed out that the preexisting linear format was specifically designed to be flexible because it allows nutrition information to be presented as a wrapped string of text that can be adapted to fit the specific dimensions of a small package. The comments suggested that the proposed ''linear'' display is not accurate because it has a ''table'' format rather than an arrangement that is linear, and it cannot be displayed as a string of wrapped text. According to the comments, the proposed linear display would not fit on many small packages for which it was intended (*i.e.,* packages that could not otherwise accommodate the tabular display for small packages, as provided in § 101.9(j)(13)(ii)(A)(*1*) (79 FR 11879 at 11979)). Other comments said that the proposed linear format would be especially problematic for products having small labels (*e.g.,* packages with 13 square inches of available labeling space) but that are not small enough to qualify for the complete exemption under § 101.9(j)(13)(i), which exempts nutrition labeling when the total surface area available to bear labeling is less than 12 square inches and no claims are made in labeling or advertising. The comments asked us to propose a revised linear format that would fit on small packages (*i.e.,* <12 square inches) or retain the preexisting linear format as an option when neither of the proposed small label formats would fit on a package. Other comments suggested that we broaden the criteria that would allow more labels to qualify for the linear and tabular formats (as provided in § 101.9(j)(13)(ii)(A)); for example, by increasing the intermediate package size from ≤40 square inches to ≤50 square inches.

(Response) We agree that the proposed linear format for small packages may not be able to fit on many small packages, such as those of confectionery products. We also acknowledge the advantage of the text wrapping feature of the preexisting linear format in providing flexibility for labels on small packages having various shapes and sizes. Consequently, we are not finalizing the requirements for the proposed linear format. Instead, we are retaining the text wrapping feature of the preexisting linear format, but adapting it to maintain consistency with the other format changes we are finalizing, *i.e.,* increasing the prominence of ''Calories'' information, removing the ''Calories from Fat'' declaration, changing ''Sugars'' to ''Total Sugars,'' including an ''Added Sugars'' declaration, modifying the mandatory vitamins and minerals, and making the abbreviated footnote ''% DV = % Daily Value'' optional for small packages. We also are providing that the actual number of servings may be listed after the ''____ servings per container'' declaration and note that ''Servings'' is an acceptable abbreviation for ''____ Servings per container'' (as provided in § 101.9(j)(13)(ii)(B)). Additionally, on our own initiative, we have revised the rule so that ''Incl. Xg added sugars'' is an acceptable abbreviation for ''includes X g of added sugars.''

However, we are concerned that some companies may be using the linear format inappropriately because we have seen the linear format used on packages that could accommodate the tabular display for small packages or on larger-size packages that could accommodate the standard format. Manufacturers should understand that the linear format is only to be used for certain size packages (as described in § 101.9(j)(13)(ii)(A)), and only if the label will not accommodate a tabular display. The linear format is more difficult to read than other formats and is not permitted for larger packages. We consider the use of a linear display as a last resort when the tabular display for small packages cannot be accommodated in the available label space (*e.g.,* when small packages with a total surface area available to bear labeling of less than 12 square inches, or 40 square inches or less and the package shape or size cannot accommodate a standard vertical column or tabular display would otherwise have to take advantage of the exemption allowing use of an address or telephone number in lieu of nutrition information). Consumers would be expected to be more likely to take a few extra moments to read a linear nutrition label than to write a letter or call the manufacturer. We do not want the linear format to be misused, so we intend to monitor the marketplace to ensure that the proper Nutrition Facts label format is used on the correct size package.

We have addressed the size and space concerns expressed in the comments for smaller packages by decreasing the prominence of the calorie declaration from our original proposal, by removing the requirement for a footnote, and permitting the abbreviated footnote ''% DV = % Daily Value'' to be optional, providing acceptable abbreviations for terms, and also permitting the text wrapping feature. Based on these spacing accommodations, we decline to increase the intermediate package size from ≤40 square inches to ≤50 square inches, as the comment suggested, because retaining the preexisting linear format and other space saving requirements would preclude the necessity of doing so.

(Comment 518) One comment stated that because foods in small packages (*i.e.,* less than 12 square inches) must bear the Nutrition Facts label if the food's label makes nutrition claims (*e.g.,* ''sugar-free'' gums), manufacturers need a Nutrition Facts label format that would fit on such packages. Otherwise, manufacturers would be prohibited from making a claim, which the comment suggested might be an unintended consequence of the final rule and adversely affect consumers (because the claim would not be available to them). Alternatively, the

comment suggested that we exempt foods in very small packages from bearing a Nutrition Facts label, even if a nutrient content claim is made or if the nutritional contribution of the food is minimal. The comment urged us to carefully consider the impact that the increase in certain type sizes and the additional ''Added Sugars'' information would have on the ability of the Nutrition Facts label to fit on very small packages.

Several comments also asked us to consider additional label formats that would be appropriate for products in small and very small packages making nutrient content claims or health claims. Some comments offered suggestions that would enable the Nutrition Facts label to fit on small and intermediate-size packages, remain legible when printed with a 6 point font size, and still ''embrace the spirit'' of our proposed rule. Specifically, the comments suggested allowing a proportional reduction of the tabular and linear formats to accommodate certain package shapes or sizes; an abbreviated format that lists fewer nutrients but would still allow a claim to be made (such as ''sugar free'' or ''calorie free''); the declaration of certain information to be voluntary; and either a telephone number, Web site, or mailing address that consumers could use to obtain more complete nutrition information (similar to the provision in § 101.9(j)(13)(i)(A)) for very small packages (*i.e.,* having less than 6 square inches of available space to bear labeling).

(Response) While we appreciate the extensive amount of time and effort that manufacturers devoted to designing alternative labels for small product packages, we disagree that such products, in general, should not be required to display a Nutrition Facts label if claims are made for the product. Depending on the particular claim and product, a variety of information may be required on the label (*e.g.,* a disclosure statement, as described in § 101.13(h)(1)) to prevent the claim from being misleading. The packages described in the comment appear to be hypothetical, as we are not aware that such packages currently exist in the marketplace.

We also decline to exempt foods in small packages that have a total surface area available to bear labeling of less than 12 square inches from bearing a Nutrition Facts label if a nutrition claim is made or if the nutritional contribution of the food is minimal. We also are continuing to allow the preexisting linear format for small packages, as described in § 101.9(j)(13)(ii)(A), which we anticipate will fit on most small confectionery packages. Furthermore, we will retain the preexisting requirement in § 101.9(j)(13)(ii)(A) that stipulates that the linear format may only be used if the label will not accommodate a tabular display.

(Comment 519) Several comments pointed out that the proposed leading requirements (*i.e.,* the vertical space between lines) differ from the preexisting leading requirements so that the proposed labels will take up more space. One comment said we could increase the amount of white space by enlarging the leading requirements. Another comment said that there was a lack of detail about the leading requirements for the information displayed in the Nutrition Facts label format shown in § 101.9(d)(12).

(Response) We agree with the comment and acknowledge an error in § 101.9(d)(1)(ii)(C) in which the leading requirements were increased. This has now been corrected in the final rule so that the original leading requirements are retained, *i.e.,* all information within the nutrition label shall utilize at least one point leading except that at least four points leading shall be utilized for the information required by paragraphs (d)(7) and (d)(8) of this section as shown in paragraph (d)(12). We allow manufacturers some degree of discretion and flexibility with respect to the leading requirements, and the label mockups that we have provided in this final regulation are for the purpose of illustration rather than to provide exact specifications. An underlying purpose of the Nutrition Facts label is to help consumers make healthful food choices, and we expect manufacturers to provide legible labels to help consumers do this.

b. Calorie conversion factors. In the preamble to the proposed rule (79 FR 11879 at 11954), we requested comments and supporting data on the extent that consumers use the caloric conversion information (*i.e.,* ''Calories per gram: Fat 9, Carbohydrate 4, Protein 4'') that may voluntarily be declared at the bottom of the footnote area of the Nutrition Facts label under § 101.9(d)(10). We stated that we may consider deleting this optional requirement in the final rule if we determine the information is not useful (id.).

(Comment 520) Some comments would prohibit the voluntary listing of caloric conversion information. These comments stated that it is too much information for consumers; its purpose in relation to the rest of the Nutrition Facts label is not readily apparent; it would require ''hands-on consumer education'' to be useful or understood; and the information is underused. One comment said that allowing the optional use of this information on the label may lead to consumer confusion because we have proposed new caloric conversion factors for certain carbohydrate subtypes.

Another comment suggested that, if we retain the optional caloric conversion information, there should also be a ''disclaimer'' or ''education statement'' indicating that the calorie values listed for fat, carbohydrate, and protein are not exact. The comment said that a disclaimer or education statement would help consumers understand that, if the grams of fat, carbohydrate, and protein that are listed on the Nutrition Facts label are multiplied by their respective caloric values (*i.e.,* 9, 4 and 4), the total may not necessarily be the same as the number of calories listed near the top of the label in the ''Calories'' declaration. The comment further suggested that such a discrepancy might cause consumer confusion. Another comment suggested the caloric information for fat, carbohydrate, and protein should be provided on a ''per ounce'' basis rather than on a ''per gram'' basis. Finally, one comment said that retaining the caloric conversion information could help consumers adjust their caloric intake if their individual calorie needs were above or below 2,000 calories per day.

(Response) We previously recognized that 9, 4, and 4 calories per gram for fat, carbohydrates, and protein, respectively, are general factors that are applicable to the majority of foods, and displaying them on the label can help consumers better understand and use the nutrition information on the label and to apply the DGA recommendations (58 FR 2079 at 2131). For example, the calorie conversion information might be useful to consumers who want to keep track of the number (or percentage) of calories they consume derived from fat and carbohydrate, or who are following certain dietary recommendations, such as for weight loss or other health reasons. Furthermore, because we are no longer requiring the number of calories from fat to be declared on the label, consumers who want this information can do their own calculations using the caloric conversion factors. We are unaware whether the caloric conversion information is underused by consumers, as suggested by one comment, and disagree that it comprises too much information, as it is displayed succinctly and is listed voluntarily. However, given the comments' concerns related to the need to conserve space on the Nutrition Facts label, we will continue to allow the caloric conversion factors to be listed voluntarily.

We disagree with the comment stating that the proposed caloric conversion factors for carbohydrate sub-types might lead to consumer confusion if the current caloric conversion information is retained. The comment did not explain this assertion. Although we proposed new caloric conversion factors for certain carbohydrate sub-types, including soluble fiber (2 calories per gram) and specific sugar alcohols (ranging from 1.6–3.0 calories per gram), consumers would not be expected to be aware of this information and would have no reason to use it because it is intended for manufacturers to use in developing product labels. Therefore, we disagree that retaining the caloric conversion information on the Nutrition Facts label would lead to consumer confusion. Furthermore, although the general conversion factors may not apply to all foods (but relatively few products would be expected to include caloric values for soluble fiber and sugar alcohols as part of the total calorie calculations), we do not consider that to be a reason to prohibit their use.

We also decline to provide a ''disclaimer'' or ''education statement'' on the label to indicate that the caloric conversion factors are approximations. The reason that multiplying the grams of fat, carbohydrate, and protein listed on the label by 9, 4, and 4 calories per gram, respectively, does not exactly add up to the number of calories listed on the label is due mainly to rounding rules that apply to the Nutrition Facts label. Rather than explain this in a footnote, however, we intend to include information about rounding as part of our planned nutrition education efforts and clarify why the caloric values of individual macronutrients may not add up to the total number of calories listed on the label.

We also do not agree that the caloric conversion factors on the label should be listed on a ''per ounce'' basis, rather than on a ''per gram'' basis, as one comment suggested. The information, if present, must be provided on a per gram basis (§ 101.9(d)(10)), which is consistent with the units that are used for declaring amounts of fat, carbohydrate, and protein on the Nutrition Facts label and therefore most likely to be useful for consumers. Furthermore, the comment did not provide data to show that ounces would be better understood or would be more useful to consumers than grams, and we have no evidence to support listing the conversion factors on a ''per ounce'' basis. We also note that the final rule no longer amends § 101.9(d)(10); we had proposed revising § 101.9(d)(10) as part of the proposed rule when we also proposed removing and reserving § 101.9(d)(9). Our proposed amendment to § 101.9(d)(10) would have removed a cross-reference to § 101.9(d)(9) and referred, instead, to a part of the Nutrition Facts label. In the supplemental proposed rule, however, we suggested text that would become a new § 101.9(d)(9) (thereby eliminating the need to reserve that paragraph). Thus, the proposed amendment to § 101.9(d)(10) is no longer necessary, and the final rule does not amend § 101.9(d)(10). (We have made a similar revision to § 101.9(d)(11) to restore a cross-reference to § 101.9(d)(9).)

With respect to the comment that said retaining the caloric conversion information could help consumers adjust their caloric intake if their individual calorie needs were above or below 2,000 calories per day, we acknowledge this is a reasonable assumption because understanding the relative amount of calories contributed by fat, carbohydrate, and protein may help consumers better comprehend and use the Nutrition Facts label, which may assist them in maintaining healthy dietary practices.

R. Compliance

Section 101.9(g) provides information about how we determine compliance with our nutrition labeling requirements, including the methods of analysis used to determine compliance, reasonable excesses and deficiencies of nutrients, and acceptable levels of variance from declared values.

1. Level of Variance Allowed for the Label Declaration of Specific Nutrients

Under our preexisting regulations, at § 101.9(g)(5), a food with a label declaration of calories, sugars, total fat, saturated fat, *trans* fat, cholesterol, or sodium shall be deemed to be misbranded under section 403(a) of the FD&C Act if the nutrient content of the composite is greater than 20 percent in excess of the value for that nutrient declared on the label. The provision provides that no regulatory action will be based on a determination of a nutrient value that falls above this level by a factor less than the variability generally recognized for the analytical method used in that food at the level involved.

The proposed rule would not change the level of variance allowed in § 101.9(g)(5).

(Comment 521) One comment suggested that we tighten the allowable variance to no more than 10 percent. The comment was concerned that the 20 percent allowable variance could result in inaccurate and misleading information going to consumers. The comment said that modern manufacturing and testing methods should allow food manufacturers to provide a more accurate representation of the nutrient content of foods.

(Response) As we stated in the preamble to the proposed rule (79 FR 11879 at 11955), we received a similar comment to the 2007 ANPRM asking us to reevaluate the level of variance permitted for nutrient content declarations. When initially determining the allowances for variability, we considered the variability in the nutrient content of foods, analytical variability inherent to test methods used to determine compliance, and statistical probability (38 FR 2125 at 2128, January 19, 1973). We also evaluated compliance procedures and found them to be statistically sound and adequate.

The comment provided no information for us to consider, such as information to show that the variability in the nutrient content of foods or analytical variability inherent in test methods used to determine compliance have decreased. Therefore, because we do not have a basis to change the level of variance permitted for the label declaration of nutrients, we decline to revise the rule as suggested by the comment.

2. Methods Used To Determine Compliance

Under our preexisting regulations, at § 101.9(g)(2), a composite of 12 subsamples, each taken from 12 different randomly chosen shipping cases are analyzed by appropriate methods as given in the ''Official Methods of Analysis of the AOAC International,'' 15th Ed. (1990) to determine compliance with the requirements in § 101.9, unless a particular method of analysis is specified in § 101.9(c). If no AOAC method is available or appropriate, we use other reliable and appropriate analytical procedures (see § 101.9(g)(2)).

The proposed rule would amend § 101.9(g)(2) to update the reference to the 19th Edition of the ''Official Methods of Analysis of the AOAC International.'' The preamble to the proposed rule (79 FR 11879 at 11913) explained that the 19th edition published in 2012 and that if a newer edition were published before we issued a final rule, we intended to finalize the rule to refer to the newer edition provided there are no substantive changes in the newer edition requiring additional comment. The Official Methods of Analysis of AOAC International, 20th Edition was

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published in 2016. The 20th Edition includes a number of new methods of analysis as well as changes to current methods. We need additional time to consider the additions and changes, and to determine if additional public comment is necessary on the 20th Edition of the AOAC Methods of Analysis. Therefore, the final rule, at § 101.9(g)(2), incorporates by reference the 19th Edition of the Official Methods of Analysis of the AOAC International.

(Comment 522) Some comments supported incorporating the 19th Edition of the AOAC Methods by reference in the final rule. Other comments suggested other alternatives. Some comments suggested that a specific edition of the AOAC Methods should not be incorporated by reference to allow companies to use future editions of the reference to meet compliance requirements. One comment stated that, given the potential limitations of the two AOAC methods for fiber identified in the proposed rule (AOAC 2009.01 and AOAC 2011.25) and the inevitable delays between adoption by AOAC of the most relevant, updated, and appropriate methods, we should incorporate all appropriate, equivalent, and validated methods into the final rule.

(Response) We decline to revise the rule to adopt the alternative approaches suggested by the comments. We note that, under the incorporation by reference regulations issued by the Office of the **Federal Register**, incorporation by reference of publication is limited to a specific edition and ''future amendments or revisions of the publication are not included'' (1 CFR 51.1(f)). Thus, under Federal regulations, we cannot incorporate by reference a specific AOAC method and all future editions of that method.

(Comment 523) Some comments questioned what we mean by ''equivalent AOAC method,'' and whether the terms mean that any other AOAC method is acceptable for determining fiber content.

(Response) We used the terminology ''equivalent AOAC method'' to mean a reliable and appropriate method which can be used for measuring dietary fiber, soluble fiber, and insoluble fiber. For example, the definition of dietary fiber requires that the fiber must contain 3 or more monomeric units. We would consider a reliable and appropriate method for dietary fiber to be one that can measure fibers with 3 or more monomeric units.

(Comment 524) Several comments suggested that AOAC 2009.01 and AOAC 2011.25 do not capture all dietary fibers. Many comments recommended that we allow for the use of all validated AOAC methods for the determination of dietary fiber. (We discuss issues related to AOAC methods in greater detail in our response to comment 299.)

(Response) In proposed § 101.9(c)(6)(i), we stated that dietary fiber content may be determined by subtracting the amount of non-digestible carbohydrates added during processing that do not meet the definition of dietary fiber from the value obtained using AOAC 2009.01, AOAC 2011.25, or an equivalent method of analysis given in the 19th edition of the AOAC methods. We stated, in proposed § 101.9(c)(6)(i)(A), that soluble fiber may be determined using AOAC 2011.25 or an equivalent method of analysis as given in the 19th edition of the AOAC Methods and stated, in proposed § 101.9(c)(6)(i)(B), that insoluble fiber may be determined using AOAC 2011.25 or an equivalent method of analysis given in the 19th edition of the AOAC Methods. Although we intended that the terms ''other equivalent methods'' refer to other AOAC methods and their AACCI counterparts, to provide clarification, the final rule omits the incorporation by reference of the specific AOAC methods in § 101.9(c)(6)(i), (c)(6)(i)(A), and (c)(6)(i)(B). Any dietary fiber declared on the label would have to meet the new definition of dietary fiber and manufacturers can measure the amount of dietary fibers in their product accurately by using a method that can measure lower molecular weight nondigestible oligosaccharides with DP 3–9. We would determine compliance by using appropriate methods, as given in the ''Official Methods of Analysis of the AOAC International,'' 19th Ed. (2012). We consider AOAC 2009.01 and AOAC 2011.25 to be reliable and appropriate methods to measure the amount of dietary fiber in a serving of a product. We consider AOAC 2011.25, as given in the ''Official Methods of Analysis of the AOAC International,'' 19th Ed. (2012), to be a reliable and appropriate method to measure the amount of soluble and insoluble fiber in a serving of a product, if separately declared. There may be other methods which manufacturers may use to measure certain fibers which can provide an accurate and consistent result. We will consider the method to use for purposes of determining compliance consistent with § 101.9(g).

3. Records Requirements

Our preexisting regulations, at § 101.9(g)(2), set forth requirements for composite sampling and analysis to determine compliance with labeling declarations. Specifically, unless a specific analytical method is identified by regulation, composites are analyzed by the appropriate AOAC method or, if no AOAC method is available or appropriate, by other reliable and appropriate analytical procedures.

In the preamble to the proposed rule (79 FR 11879 at 11956), we noted that, for certain nutrients subject to the proposed rule, there is no AOAC official method of analysis or other reliable or appropriate analytical procedure that is available for us to verify the amount of the declared nutrient on the Nutrition Facts label and ensure that the declared nutrient amount is truthful, accurate and complies with all applicable labeling requirements. The preamble to the proposed rule (79 FR 11879 at 11956) stated that there is no suitable analytical procedure available to measure the quantity of: (1) Added sugars (when a food product contains both naturally occurring sugars and added sugars and for specific foods containing added sugars, alone or in combination with naturally occurring sugars, where the added sugars are subject to non-enzymatic browning and/or fermentation); (2) dietary fiber (when a food product contains both non-digestible carbohydrate(s) that meets the proposed definition of dietary fiber and non-digestible carbohydrate(s) that does not meet the definition of dietary fiber); (3) soluble fiber (when a mixture of soluble fiber and added nondigestible carbohydrate(s) that does not meet the definition of dietary fiber are present in a food); (4) insoluble fiber (when a mixture of insoluble fiber and non-digestible carbohydrate(s) that does not meet the definition of dietary fiber are present in a food); (5) vitamin E (when a food product contains both RRR- α-tocopherol and *all rac*-α-tocopherol acetate); and (6) folate (when a food product contains both folate and folic acid).

Under our preexisting regulations, at § 101.9(g)(9), we may permit the use of an alternative means of compliance or additional exemptions when it is not technologically feasible, or some other circumstance makes it impracticable, for firms to comply with the requirements of § 101.9. Under § 101.9(g)(9), firms must submit a written request to us for the use of an alternative means of compliance or for a labeling exemption.

The proposed rule would establish an alternative approach for assessing compliance of the declared amount of certain nutrients when there is no suitable analytical method available to measure the nutrient's quantity as

declared on the label or in labeling. Specifically, the proposed rule, at proposed § 101.9(g)(10) and (g)(11), would require the manufacturer to make and keep records that are necessary to verify the declaration of: (1) The amount of added sugars when both naturally occurring and added sugars are present in a food (in § 101.9(c)(6)(iii)); (2) the amount of added non-digestible carbohydrate(s) that does not meet the proposed definition of dietary fiber when the dietary fiber present in a food is a mixture of non-digestible carbohydrates that do and that do not meet the definition of dietary fiber (in § 101.9(c)(6)(i)); (3) the amount of added soluble non-digestible carbohydrate(s) that does not meet the proposed definition of dietary fiber when the soluble dietary fiber present in a food is a mixture of soluble non-digestible carbohydrates that do and that do not meet the definition of dietary fiber (in § 101.9(c)(6)(i)(A)); (4) the amount of added insoluble non-digestible carbohydrate(s) that does not meet the proposed definition of dietary fiber when the insoluble dietary fiber present in a food is a mixture of insoluble non-digestible carbohydrates that do and that do not meet the definition of dietary fiber (in § 101.9(c)(6)(i)(B)); (5) the amount of *all rac*-α-tocopherol acetate added to the food and RRR-α-tocopherol in the finished food when a mixture of both forms of vitamin E are present in a food (in § 101.9(g)(10)(i)); and (6) and the amount of folic acid added to the food and the amount of folate in the finished food when a mixture of both forms are present in a food (in § 101.9(g)(10)(ii)). In the preamble to the proposed rule (79 FR 11879 at 11956), we explained that the manufacturer is in the best position to know which of its records provide the documentation required under the circumstances described for us to determine compliance. These records could include one or more of the following: Analyses of databases, recipes or formulations, or batch records. We stated that most manufacturers should already have the type of records needed to validate the declared amount of these nutrients and that the proposed records requirements provide flexibility in what records the manufacturer makes available to us to verify the declared amount of these nutrients for a particular marketed product (id.).

The proposed rule, at proposed § 101.9(g)(11), also would require that records be kept for a period of 2 years after introduction or delivery for introduction of the food into interstate commerce and that such records be provided to us upon request during an inspection for official review and copying or other means of reproduction. The proposed rule also stated that records could be kept either as original records, true copies (such as photocopies, pictures, scanned copies, microfilm, microfiche, or other accurate reproductions of the original records), or electronic records in accordance with 21 CFR part 11.

(Comment 525) Many comments agreed with the proposed recordkeeping requirements. However, other comments objected to the proposed recordkeeping requirements. Some comments said that our compliance program for nutrition labeling should be based on the validation of nutrient declarations through analytical methods and not through recordkeeping. Other comments said that compliance should be based on objective, analytical measures to yield consistent labeling practices across the food industry. Others comments said that the proposed recordkeeping requirements could invite unethical manufacturers to provide inaccurate information about the quantity of nutrients in a serving of their product.

(Response) As discussed in the preamble to the proposed rule (79 FR 11879 at 11956), for certain nutrients, there are no official methods of analysis or other reliable or appropriate analytical procedures that are available to verify the amount of the declared nutrient on the Nutrition Facts label. In the absence of such methods, there needs to be some means for determining compliance, and so we proposed recordkeeping as an alternative approach for assessing compliance of the declared amount of certain nutrients. While the amount of most other nutrients in Nutrition Facts can be verified analytically, for those nutrients whose amounts cannot be determined analytically, recordkeeping enables FDA to determine compliance with § 101.9(g). Regarding the potential for encouraging manufacturers to provide inaccurate information to FDA, we note that all nutrient declarations must be truthful and not misleading under sections 403(a)(1) and 201(n) of the FD&C Act. Thus, whether determined analytically or through calculations documented in appropriate records, manufacturers are obligated to provide nutrient information that is not false or misleading.

(Comment 526) Several comments said that it would be very difficult to obtain and retain the information required by FDA. Some comments noted that the number of product formulations can be greater than 20,000 for certain manufacturers and that they would need to create systems and dedicate additional resources to create and maintain appropriate records on a large scale. Other comments said that manufacturers typically get ingredients from suppliers in an extensive supply chain and that many ingredients also contain multiple ingredients themselves. Suppliers may not have the information themselves, or the information for the formulations could be proprietary. Additionally, nutrient information could be provided in ranges, and manufacturers would be unable to determine or verify the specific amounts of certain nutrients analytically.

(Response) Although some manufacturers could have a large number of foods that contain nutrients that would necessitate recordkeeping to verify amounts, we do not agree that determining the nutrient composition of a food and recording that information would present undue difficulty for manufacturers. On the contrary, knowledge of what ingredients and nutrients are in a food and providing that information truthfully to consumers is a basic requirement for food producers. Manufacturers, even those who produce large amounts of food products, have experience with determining nutrient content of the food they produce, and the maintenance of records of nutrient content, either written or electronic. Regarding obtaining information from ingredient suppliers, manufacturers are well suited to work with suppliers to ensure that proper information is communicated throughout the supply chain. Ingredient suppliers are obliged to have knowledge of the contents of ingredients they provide to food manufacturers and this information will need to be properly communicated. Manufacturers may be able to choose suppliers that provide appropriate information as to the contents of their ingredients or be able to ask their ingredient suppliers for nutrient information.

(Comment 527) Some comments suggested that the required approach should be flexible and not mandate a specific type of record. The comments indicated that manufacturers should be able to substantiate using the records they believe best accomplish the validity of nutrient information. The comments stated that we did not need access to manufacturing records and that other methods, such as database information or an explanation from a manufacturer, would suffice.

(Response) Manufacturers will be responsible for the type of records they maintain and are not required to

produce any specific form or document for verification purposes. Records used to verify nutrient content could include various types of batch records providing data on the weight of certain nutrient contributions to the total batch, records of test results conducted by the manufacturer or an ingredient supplier, certificates of analysis from suppliers subject to initial and periodic qualification of the supplier by the manufacturer, or other appropriate verification documentation that provide the needed assurance that a manufacturer has adequately ensured the food or ingredients comply with labeling requirements. The records submitted for inspection by FDA would only need to provide information on the nutrient(s) in question. Information about other nutrients can be redacted if necessary to ensure confidentiality of a food product formulation.

(Comment 528) Several comments addressed our legal authority to require recordkeeping as described in the proposed rule.

(Response) We address these comments in part II.C.4.

(Comment 529) Some comments expressed concern that proprietary information in recipes and formulations could be divulged and said that the ability to retain and claim the proprietary nature of product formulations is essential to staying competitive in the marketplace. Other comments suggested that we clarify that the recordkeeping requirements will not require access to proprietary information, such as recipes and formulations. In addition, the comments recommended that we specify what level of information and types of documents are required to meet the recordkeeping requirements. Several comments requested that manufacturers be permitted to develop a stand-alone document that articulates the basis for the declaration of added sugars in a product. Other comments recommended that, if we finalize the recordkeeping requirements and require the copying of records, we address the security of the information coming from inspections and the protection of confidential information.

(Response) The final rule does not require a specific document to be retained nor does it require information on proprietary recipes or overall formulations. Instead, the recordkeeping requirements seek specific content information for certain nutrients, and this information can be provided in various forms. For example, information in some batch records could include data on the total batch weight of the production of a particular food and also provide data on the weight of certain nutrient contributions to the total batch. With these types of data, calculations can be made to determine nutrient content for individual foods or servings of a food. Documentation of this type would not reveal any proprietary recipes or formulations and would be limited to specific nutrient information. Information about the nutrient content of the ingredients of a food product could be acquired from ingredient suppliers subject to initial qualification and periodic requalification by the manufacturer, and this type of information on quantitative source amounts can be included in the batch records.

Furthermore, even if a manufacturer's records contained confidential commercial information or trade secret information or a manufacturer believes that certain information should be protected from public disclosure, we note that there are safeguards to protect against public disclosure of that information and mechanisms that a manufacturer can use to assert that certain information should be protected from disclosure. As we stated in the preamble to the proposed rule (79 FR 11879 at 11957), we would protect confidential information from disclosure, consistent with applicable statutes and regulations, including 5 U.S.C. 552(b)(4), 18 U.S.C. 1905, and part 20 (21 CFR part 20). For example, our regulations pertaining to disclosure of public information, at part 20, include provisions that protect trade secrets and commercial or financial information which is privileged or confidential. If a manufacturer keeps proprietary recipe information in its records, it should mark the information as such before providing the records to us upon request.

(Comment 530) One comment expressed concerns that allowing for recordkeeping as a way to verify the amount of nutrients such as added sugar in some products would encourage those manufacturers to provide false reporting of the added sugar content of their products.

(Response) We note that having a false declaration on the label is a violation of section 403(a)(1) of the FD&C Act. Providing false information in records to the Agency may also be a potential criminal violation under 18 U.S.C. 1001. Under 18 U.S.C. 1001, whoever, in any matter within the jurisdiction of the executive, legislative, or judicial branch of the Government of the United States, knowingly and willfully: (1) Falsifies, conceals, or covers up by any trick, scheme, or device a material fact; (2) makes any materially false, fictitious, or fraudulent statement or representation; or (3) makes or uses any false writing or document knowing the same to contain any materially false, fictitious, or fraudulent statement or entry may be subject to a fine or imprisonment.

(Comment 531) Some comments disagreed with the proposed requirement to keep records for at least 2 years after a food's introduction into interstate commerce. The comments said manufacturers would have to keep track of an additional data point (the date on which the food is actually shipped) as opposed to the date on which it is manufactured. The comments said that shipping dates can vary, even for foods from the same batches, and could occur months after manufacture, and this could result in extremely divergent record maintenance timeframes for foods.

Furthermore, some comments said that is unclear whether the term ''food'' is intended to refer to a particular batch of food or to an individual food.

Other comments suggested that 2 years is a long time for foods with very short shelf lives. Some comments noted that the Seafood Hazard Analysis and Critical Control Points (HACCP) regulations allow for a 1-year record retention period for refrigerated products and a 2 year period for frozen, preserved, or shelf-stable products. The comments suggested that, similarly, the 2 year requirement for recordkeeping related to nutrition labeling should be limited to frozen, preserved, or shelf-stable products and that a shorter period of 1 year should be allowed for maintenance of records for refrigerated and perishable foods.

(Response) We recognize that there can be a wide variation of manufacturing practices, shipping practices, and shelf lives among packaged foods. We believe, however, that it is more practical to establish a single recordkeeping period rather than establish different recordkeeping periods for different products or for different manufacturing or shipping practices. It would be more difficult for FDA to establish a compliance program for one segment of the regulated industry that starts the recordkeeping process when the food is made and a different compliance program for another segment of the industry that starts the recordkeeping process when the food is shipped. Likewise, for manufacturers who make several food products, it may be easier for them to use the same recordkeeping period for all products rather than use different recordkeeping periods for different products. Therefore, we have designed a

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Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets

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Abstract

The fate of cellular DNA during the standard purification steps of the sugar manufacturing process from conventional and transgenic sugar beets was determined. Indigenous nucleases of sugar beet cells were found to be active during the first extraction step (raw juice production) which was carried out at 70°C. This and the consecutive steps of the manufacturing process were validated in terms of DNA degradation by competitive PCR of added external DNA. Each step of the process proved to be very efficient in the removal of nucleic acids. Taken together, the purification steps have the potential to reduce the amount of DNA by a factor of $>10^{14}$, exceeding by far the total amount of DNA present in sugar beets. Furthermore, the gene products of the transgenes neomycin phosphotransferase and BNYVV (rhizomania virus) coat protein CP21 were shown to be removed during the purification steps, so that they could not be detected in the resulting white sugar. Thus, sugar obtained from conventional and transgenic beets is indistinguishable or substantially equivalent with respect to purity. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: *Beta vulgaris*; Transgenic sugar beets; Sugar purification; Competitive PCR; Rhizomania

1. Introduction

The development of transgenic varieties of various plants, and also sugar beets, had become feasible by application of selectable marker gene introduction with the Ti-plasmid derived vectors due to the pioneering work of Bevan et al. (1983) and Herrera-Estrella et al. (1983). For the generation of transgenic sugar beets (*Beta vulgaris*), an improved method using stomatal guard cells has recently been reported (Hall et al., 1996). Since then, numerous transgenic lines have been constructed and their usefulness demonstrated in outdoor plantations.

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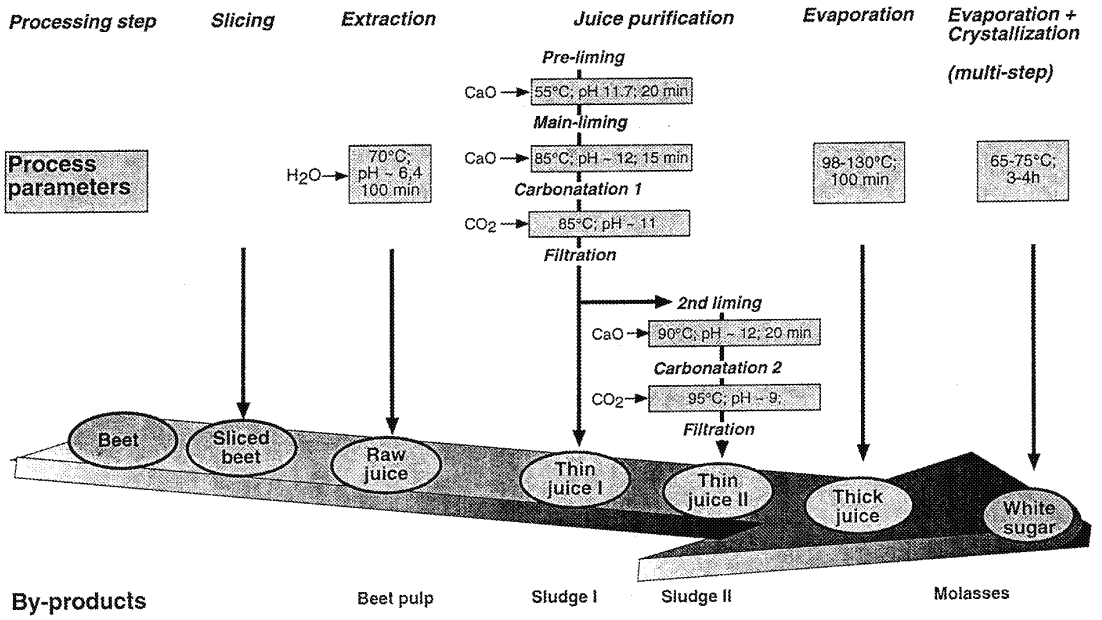

Fig. 1. Principal steps of sugar production from sugar beets.

One major goal in generating transgenic varieties is the establishment of resistance against plant viruses. The first report on this made use of the introduction and expression of virus coat protein genes in plant cells (Abel et al., 1986). The major virus related disease of sugar beets is rhizomania caused by the beet necrotic yellow vein virus (BNYVV). The genetic map of the multipartite genome of this virus has been reviewed (Richards and Tamada, 1992). The introduction of a gene cassette coding for the *cp*21 gene product (coat protein, CP21) of BNYVV under the control of the cauliflower mosaic virus promoter into cells of *B. vulgaris* resulted in plants resistant to BNYVV infection (Kallerhoff et al., 1990; Ehlers et al., 1991). The addition of this gene cassette to the genome of *B. vulgaris* was supported by coupling the CP21 construct to a neomycin resistance gene (*aphA*) allowing selection by G418 treatment of cultivars during the early stages of their cultivation.

The first successful outdoor plantations of transgenic virus resistant sugar beet cell lines raised the question about the fate of genetic material and proteins during the sugar manufacturing process.

Sugar is recovered from beet by a multistep extraction and purification procedure (Fig. 1). This includes slicing of washed beets (to 'cossettes') followed by extraction with water at elevated temperature (70°C) for about 100 min. The raw juice obtained is clarified by two consecutive steps comprising CaO addition (liming) and subsequent carbonatation. The material precipitated thereby (sludge) is removed by filtration to yield a so-called thin juice. It is concentrated by evaporation first to thick juice and then further to a crystal magma from which high purity sugar is recovered by centrifugation. The evaporation of thin juice to thick juice is carried out in a multi-effect evaporator working at a temperature range of 98–130°C.

The objective of this study was to analyse intermediate and end products of the standard sugar recovery process for DNA using the ADP-glucose pyrophosphorylase gene (AGPase, *agp*, Smith-

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White and Preiss, 1992) as a general marker for sugar beet DNA, and the genes for the BNYVV coat protein (*cp*21) and neomycin phosphotransferase (*aphA*) and their respective gene products as specific markers for transgenic beet DNA and proteins. Furthermore, the potential of each principle processing step to remove DNA was validated with added pUC18 DNA (Yanisch-Perron et al., 1985). The methods applied comprised agarose gel electrophoresis, hybridisation methods, competitive PCR and immunological as well as enzymatic methods.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Cloning experiments and plasmid preparations were carried out in *E. coli* JM109. Strains with plasmids were grown in 2 × YT liquid medium or on 2 × YT agar plates (Sambrook et al., 1989) supplemented with 100 μg ml^{-1} ampicillin at 37°C.

2.2. DNA preparation, DNA manipulation and cell transformation

Plasmid preparations from *E. coli* were performed by the method of Kieser (1984). Large scale plasmid preparation was done by using the Qiagen plasmid giga kit (Qiagen, Hilden, Germany). To isolate genomic DNA, frozen beets or frozen cossettes (3 g) were chopped up in liquid nitrogen and homogenised for 2 min in 1 vol Kirby mix (1% triisopropylnaphthalenesulfonic acid, Na salt, 6% 4-aminosalicylic acid and 6% phenol in 50 mM Tris–HCl, pH 8.3; Sambrook et al. (1989)) and 2 vol phenol/chloroform. After centrifugation, the supernatant was reextracted with 1 vol phenol/chloroform and the DNA precipitated with ethanol. Finally, the DNA was resuspended in TE buffer (Sambrook et al., 1989) and dialysed in the same buffer. Raw juice (1 ml), thin juices (1 ml), samples of sludge I and II (1 g resuspended in 1 ml TE buffer), thick juice (1 ml) and white sugar (3 g diluted in 3 ml water) were treated with 0.5 ml phenol/chloroform and centrifuged at 6000 × *g* for 15 min. The supernatant was dialysed in a buffer containing PEG 6000 (5 mM Tris–HCl, pH 8.8, 0.5 mM EDTA, 5 mM NaCl, 3.5% PEG 6000) and hereby 10-fold concentrated. Finally, the DNA was purified via the Qiaquick-spin PCR purification kit of Qiagen. All other DNA manipulations were carried out as described elsewhere (Sambrook et al., 1989).

2.3. Quantitative PCR

The competitive PCR was carried out as already described (Gilliland et al., 1990; Ferre, 1992). In a total volume of 40 μl DNA, 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the four deoxynucleotide triphosphates (Pharmacia, Uppsala, Sweden), 0.5 μM of each forward and reverse primer and 2.5 U *Taq* DNA polymerase (Pharmacia) were added. The first step was for 1 min at 94°C, followed by 30 cycles of denaturation for 30 s at 92°C, annealing for 1 min (S672/S673: 59°C, S674/S675: 59°C, S700/S701: 53°C, S708/S709: 50°C) and extension for 2 min at 72°C (thermal cycler PTC-200, MJ Research, Watertown, USA). The PCR fragments were separated by electrophoresis through 1% agarose gels, visualised by UV light after ethidium bromide staining, documented and quantified. A videocamera and the software package of Cybertech (Cybertech DS1, Cybertech, Berlin, Germany) was used to determine the equivalence concentration where standard and target DNA concentration were identical. The competitor DNA was added at concentrations ranging from 5 ag to 500 fg. This corresponded to about 1.5 and 150 000 molecules.

2.4. Primer, target and competitor DNA

The plasmids, primers and fragment sizes obtained by PCR are listed in Table 1. The plasmids pJKS224, pJKS230 and pJKS219 were generated by amplification of fragments of *agp*, *cp*21 and *aphA* from transgenic beet DNA and inserted between the *Pvu*II sites of pUC18. The plasmids with the competitor DNA were generated by deleting a *Pvu*II fragment from pUC18

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Table 1
Target and competitor DNAs

Primer sequence	T_{anneal} (°C)	Target DNA		Competitor DNA	
		Plasmid/gene	Fragment size (bp)	Plasmid	Fragment size (bp)
S672: ATACGCAAACCGCCTCTCC S673: ATACCGCATCAGGCGCCAT	59	pUC18	434	pADI2.2	800
S700: TGGCAGAAGCACATTGA-CAC S701: TTGGGAGGCTGTTGTG-TAAG	53	*agp* (pJKS224)	776	pJKS227	600
S708: CCAGGGACTTCAGCAGGTG S709: CAGGAACCGCAGGAGTGGA	50	*cp*21 (pJKS230)	177	pJKS232	350
S674: CTCTGATGCCGCCGTGTTC S675: GCCCATTCGCCGCCAAGCT	59	*aphA* (pJKS219)	618	pJKS222	800

The used primers and the sizes of the PCR fragments after quantitative PCR are listed. The plasmids which contain the target PCR fragments are shown in brackets.

(pADI2.2), a *Eco*RV/*Nsi*I fragment from pJKS224 (pJKS227), a *Nsi*I/*Sca*I fragment from pJKS230 (pJKS232) and a *Pst*I/*Sph*I fragment from pJKS219 (pJKS222) and replacing them with *Hae*II fragments from bacteriophage λ. It was verified that the constructed internal standard (competitor) DNAs had comparable efficiencies of amplification as the appropriate pUC18-based target DNAs using the method described by Scadden et al. (1992). The 5 pg target and competitor DNA were independently analysed.

2.5. Hybridisation of DNA

Total genomic DNA was isolated and digested with restriction endonucleases. After electrophoresis, the DNA was transferred onto a nylon membrane (Immobilon P, Millipore, Eschborn, Germany) and hybridised with the cloned PCR fragments of the target DNA, labelled by using a non-radioactive DNA labelling and detection kit (Boehringer, Mannheim, Germany). Hybridisation was carried out at 68°C in hybridisation buffer as described by the manufacturer.

2.6. Immunological methods

Neomycin phosphotransferase and CP21 protein were detected by sandwich ELISAs using a biotin–streptavidin amplification system (5'Prime 3'Prime Inc., Boulder, USA). Absorbance values at 405 nm were read in a microplate reader (model 3550, Bio-Rad, Munich, Germany).

3. Results and discussion

3.1. DNA disappears from cossettes during extraction

The plant material used (about 25–30 kg of beets) was collected from different field trials and subjected to standard processing in a pilot plant and analysed. Conventional beets free of BNYVV (A) and conventional BNYVV-infected beets (B) served as controls. Beets of transgenic varieties (C) from BNYVV free areas were compared with the controls.

Genomic DNA from fresh sugar beet cossettes could be prepared by standard DNA extraction

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methods based on phenol extraction and ethanol precipitation (Section 2). However, DNA could not be detected in ethidium bromide (EtBr) stained agarose gels when this method was applied to post-extraction beet cossettes (pulp) or raw juice either. Southern blot analysis of these gels using a labelled cDNA of ADP-glucose pyrophosphorylase as a reference for genomic sequences of *B. vulgaris* cells and fragments from *aphA* or *cp*21 genes in case of transgenic beets again gave negative results (data not shown). Obviously, DNA disappeared during the process of juice extraction at 70°C for unknown reasons.

3.2. Nucleases from beet extracts degrade DNA in raw juice

When purified nucleic acid from fresh sugar beet cossettes was added to raw juice at 70°C, a quick degradation of DNA was observed by EtBr-stained agarose gel electrophoresis (Fig. 2A). This pointed towards the presence of DNA degrading activities, e.g. nucleases in the raw juice.

To corroborate this point, 250 μg ml^{-1} pUC18 DNA were added to fresh raw juice samples and incubated for the periods indicated in Fig. 2B. The amount of pUC18 DNA added by far exceeded the calculated amount of $\sim$10 μg ml^{-1} whole cellular DNA, assuming total lysis of all beet cells. Under these conditions, the added pUC18 DNA was shown to be degraded within minutes.

The rate of this DNA degradation could be shown to be temperature dependent (Fig. 2C) having low efficacy at 4°C, a slow degradation at 37°C but a high degradation activity at 70°C. Protein denaturation measures such as heating of raw juice at 95°C for 10 min or phenol extraction of raw juice resulted in the protection of added beet genomic DNA or pUC18 DNA from degradation (data not shown).

3.3. Degradation of the agp, aphA and cp21 DNA during the sugar recovery process steps as analysed by PCR

The *B. vulgaris* genomic DNA content both in raw juice and pulp (sugar beet cossettes after

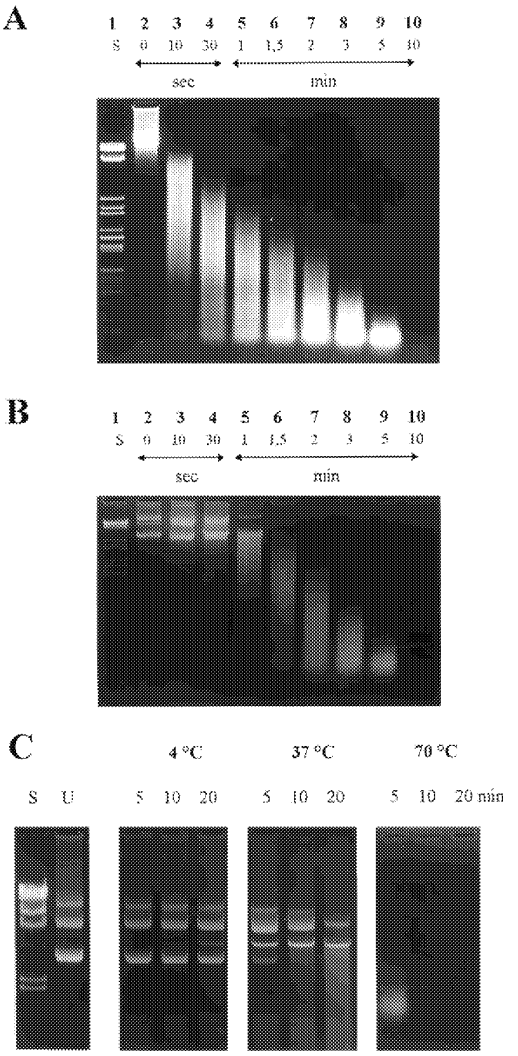

Fig. 2. Degradation of sugar beet chromosomal DNA (A), pUC18 DNA (B) and pUC18 DNA under various temperatures (C) in sugar beet raw juice. Chromosomal (A) or pUC18 DNA (B, C) were added to 500 μl raw juice from conventional beets free of BNYVV at a final concentration of 250 μg ml^{-1} at 70°C (A, B) or at 4, 37 and 70°C (C). Samples (20 μl), which were immediately extracted in the same volume of phenol/chloroform solution, were taken at the indicated times. Of the samples, 10 μl were separated by agarose gel electrophoresis; λ DNA cut with *Bgl*I (S) and uncut pUC18 DNA (U) were used as markers.

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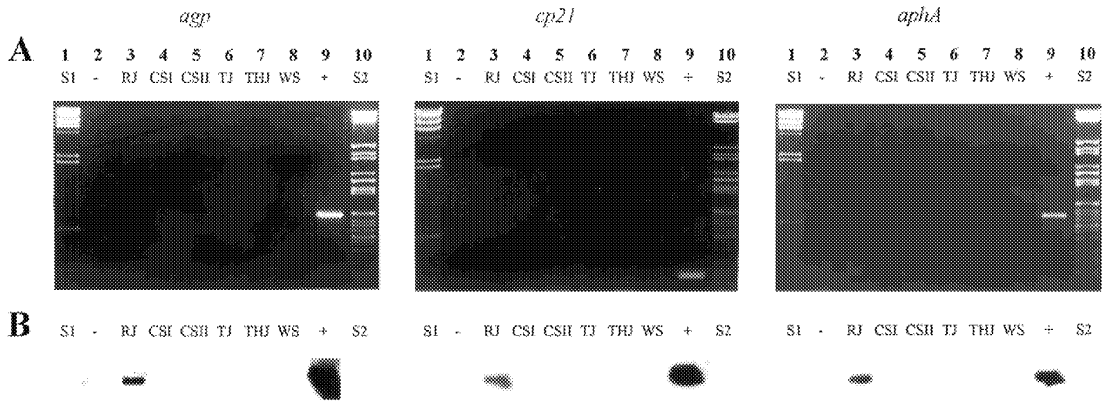

Fig. 3. Analysis of the *agp*, *cp*21 and *aphA* genes in raw juice (RJ), carbonatation sludge I (CSI), carbonatation sludge II (CSII), thin juice (TJII), thick juice (THJ) and white sugar (WS) from transgenic beets. The DNA was prepared as described in Section 2. DNA solutions (5 μl) were subjected to PCR using the primer set S700/S701 for the *agp* gene, S708/S709 for the *cp*21 gene and S674/S675 for the *aphA* gene; 100 pg of the appropriate plasmids containing the different target DNAs (pJKS224: *agp*, pJKS230: *cp*21 and pJKS219: *aphA*) were added as positive PCR controls (lane 9, +), the negative control was without DNA (lane 2, −). PCR reactions (10 μl) were separated via agarose gel electrophoresis (A), the DNA transferred to a nylon membrane and hybridised to DIG-labelled target DNA (B).

extraction of raw juice) was below detection limit of conventional methods such as Southern blot analysis. Therefore, the more sensitive PCR analysis was applied to these materials as well as to samples from the latter process steps.

Direct PCR analysis of raw juice samples with added pUC18 DNA gave only barely detectable signals, pointing towards factors in raw juice preventing efficient PCR amplification. Therefore, raw juice samples and those from subsequent processing steps were purified by phenol extraction followed by dialysis and DNA affinity chromatography. pUC18 DNA added to such purified samples could then be amplified as efficiently as the control with buffer (data not shown).

In samples from all processing steps, from raw juice to white sugar, from conventional as well as transgenic beets, DNA could not be detected using PCR with primers for *agp*, *aphA* and *cp*21 DNA (Section 2) followed by agarose gel electrophoresis and EtBr-staining (Fig. 3A). The more sensitive Southern blot hybridisation with digoxigenin-labelled DNA of the target DNAs gave clearly recognisable signals in PCR samples from raw juice only, but in none of the consecutive products. Chromosomal *agp* DNA was detected in raw juice from conventional and transgenic beets whereas the specific transgenic markers were found in raw juice from the respective beets only (Fig. 3B).

Quantification of DNA was performed by competitive PCR analysis according to Piatak et al. (1993). This comprises the comparison of the amounts of PCR products resulting from the co-amplification of a target sequence and an added internal standard of known concentration and recognisable by the same primer pair. Competitive plasmids for *cp*21, *aphA* and *agp* sequences as well as for pUC18 DNA were constructed (Section 2). The internal standard (competitor) DNAs were determined to have comparable amplification efficiencies as the appropriate pUC18-based target DNAs using the method described by Scadden et al. (1992).

The DNA content in raw juice being too low for proper quantification, it had to be concentrated 10-fold by DNA-affinity chromatography. Thereby, for each of the three gene fragments analysed equivalence concentrations of 2×10^4 molecules per 1 ml raw juice could be determined. This corresponds to about 5–10 fg of the constructed plasmids.

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Assuming a triploid genome (3 pg DNA per cell), a cell content of 10^6 cells in 1 g beet material (microscopically determined) and as 1 kg of sugar beets results in about 1.15 l of raw juice, this would mean a 100-fold reduction of the gene fragments (copy number basis). However, as the methodology is based on copy number comparison and the competitor DNA used is much smaller than chromosomes, the actual fragmentation of chromosomal DNA is to be expected to be much higher. The quantification of *agp* is shown as an example in Fig. 4.

3.4. DNA reduction potential of various sugar recovery process steps using added pUC18 DNA

The low number of DNA fragments detected in raw juice prompted us to validate all steps of the sugar recovery process for their potential to degrade or remove DNA.

For the first carbonatation step pUC18 DNA was added at a high dosage of 250 μg ml^{-1} to heat inactivated raw juice and liming and carbonatation was performed according to standard procedure. After filtration, samples of juice (so-called thin juice I) and sludge (sludge I) were retained and the main portion of juice subjected to a second liming and carbonatation treatment resulting in thin juice II and sludge II. The samples of thin juice I and II were dialysed and the DNA concentrated by affinity chromatography. Competitive PCR showed a 10^3-fold reduction of pUC18 DNA in the first and a 10^5-fold reduction in the second carbonatation step. Samples of sludge I and II were extracted, each with the same volume of water, dialysed and concentrated by affinity chromatography. They were shown by PCR to be free of DNA.

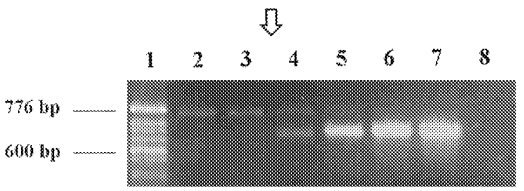

Fig. 4. Quantitative PCR of the *agp* DNA in raw juice: 15 μl of the 10 times concentrated and purified raw juice in the presence of 750 ag (lane 2), 5 fg (lane 3), 10 fg (lane 4), 30 fg (lane 5), 50 fg (lane 6) and 500 fg (lane 7) of competitor DNA pJKS227, respectively 220, 1470, 2940, 8800, 14 700 and 147 000 copies of pJKS227 were subjected to PCR. A negative control (lane 8) did not contain any DNA; 10 μl of the PCR reactions were separated via agarose gel electrophoresis and analysed as described in Section 2; λ DNA cut with *Bgl*I was used as molecular weight marker. The arrow indicates the equivalence concentration.

The results were verified by adding 0.250 μg ml^{-1} pUC18 DNA to heat inactivated raw juice. The competitive PCR confirmed a 10^3-fold reduction of pUC18 DNA in the first carbonatation step and showed this factor independent from the actual amount of DNA present. After the second carbonatation step no DNA was found, i.e. the DNA concentration was reduced by a factor of at least 10^5 in the second carbonatation. Again, there was no DNA to be detected in the sludge samples. In summary, during juice purification residual DNA fragments from raw juice will be reduced at least 10^8-fold.

The next step in the sugar recovery process is the multistep evaporation of thin juice II at a temperature range of 98–130°C and a residence time of $\sim$30 min to produce a thick juice. To simulate this step in the laboratory, a thin juice II sample with 250 μg ml^{-1} pUC18 DNA added was autoclaved at 121°C for 30 min. Thereby, a 10^3-fold reduction of pUC18 DNA concentration was shown by competitive PCR.

The last purification step in the sugar recovery process is crystallisation by evaporation of thick juice at a temperature of about 70°C followed by separation and washing of crystals in a sieve-basket centrifuge. This process step was carried out in the laboratory after adding 250 μg ml^{-1} pUC18 DNA to thick juice and evaporating to crystallisation. It was, however, not possible to wash the crystals in the laboratory centrifuge. Nevertheless, only about one-tenth of the DNA added could be found again.

The DNA degrading potential of nucleases in the raw juice was tested by adding pUC18 DNA (0.025 and 2.5 μg ml^{-1}) at 70°C. Samples taken at different times up to 120 min were analysed by competitive PCR. As shown in Fig. 5, pUC18 DNA was rapidly degraded within 15 min, reducing the copy numbers of intact target sequence by

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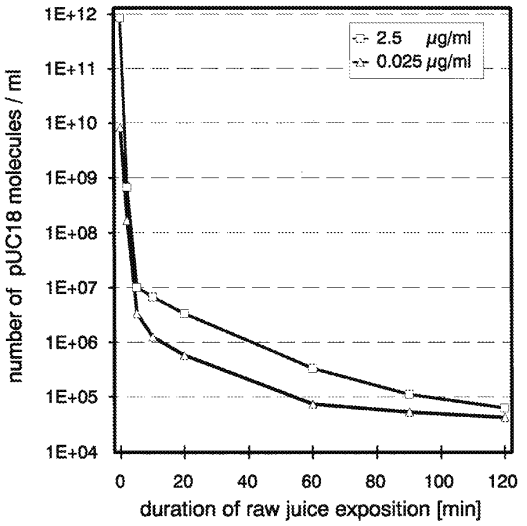

Fig. 5. Decrease of pUC18 DNA molecules in sugar beet raw juice. pUC18 DNA was added to 500 μl raw juice from conventional beets free of BNYVV at final concentrations of 0.025 (△) and 2.5 μg ml^{-1} (□); 20 μl samples were taken at the indicated times, immediately extracted with 20 μl phenol/chloroform and purified via a Qiagen column (Qiagen, Hilden, Germany). The amount of pUC18 molecules per μl raw juice was quantified via competitive PCR using the standard DNA pADI2.2.

a factor of about 10^5 (in the 2.5 μg ml^{-1} sample) followed by a slowing down of the degradation rate. This was found to be not due to inactivation of nucleases during the incubation period as a preincubation of raw juice for 120 min at 70°C led to similar degradation kinetics (not shown). It is assumed that the nuclease activity decreases at low DNA concentrations and increasing DNA fragmentation.

The factor of overall efficacy of DNA elimination under standard process conditions can be calculated to about 10^{14}. These activities include nucleolytic degradation in raw juice, irreversible adsorption on sludge, precipitation, denaturation and presumably hydrolysis due to alkaline pH and high temperature in the carbonatation steps, hydrolysis at the very high temperature during the evaporation step and exclusion of DNA from sugar crystals in the last step. The non-enzymatic steps should be independent of DNA concentrations and therefore capable of completely removing the low amounts of DNA left in the raw juice.

The reduction of biologically active DNA should even be greater as the DNA was considerably reduced in size in the raw juice and, later on, denatured to single-stranded DNA. This is because only small parts of the genes or pUC18 DNA were amplified and the actual size of the fragments may have even been smaller than the PCR fragments due to the extension of overlapping small fragments by *Taq* polymerase.

3.5. Proteins are removed during juice purification

The fate of the gene products of the transgenes was also looked at, e.g. neomycin phosphotransferase and BNYVV coat protein CP21. Applying ELISA methods for detection of neomycin phosphotransferase, 4×10^{-8} g ml^{-1} could be detected in raw juice from transgenic beets (C). Quantification of CP21 by the same technique showed that raw juice samples from BNYVV-infected conventional beets (B) contained 5×10^{-5} g ml^{-1} CP21, i.e. 10^3 times more than samples from BNYVV-free transgenic beets (C) which contained 3×10^{-8} g ml^{-1}. No AphA ($< 10^{-10}$ g ml^{-1}) or CP21 ($< 5 \times 10^{-9}$ g ml^{-1}) was found in pulp, thin juices, thick juice or white sugar from transgenic beets. This shows that proteins are efficiently removed during the juice purification steps.

In summary, extraction and purification steps of the standard sugar production process are very efficient in removal of nucleic acids and proteins irrespective of their origin. Consequently, the product, white sugar, is indistinguishable from its source: the transgenic beet varieties or conventionally bred controls.

References

Abel, P.P., Nelson, R.S., De, B., Hoffmann, N., Rogers, S.G., Fraley, R.T., Beachy, R.N., 1986. Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science 232, 738–743.

Bevan, M.W., Flavell, R.B., Chilton, M.-D., 1983. A chimaeric antibiotic resistance marker gene as a selectable marker for plant cell transformation. Nature 304, 184–187.

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Ehlers, U., Commandeur, U., Frank, R., Landsmann, J., Koenig, R., Burgermeister, W., 1991. Cloning of the coat protein gene from beet necrotic yellow vein virus and its expression in sugar beet hairy roots. Theor. Appl. Genet. 81, 777–782.

Ferre, F., 1992. Quantitative or semi-quantitative PCR: reality versus myth. PCR Methods Appl. 2, 1–9.

Gilliland, G., Perrin, S., Blanchard, K., Bunn, H. F., 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87, 2725–2729.

Hall, R.D., Riksen-Bruinsma, T., Weyens, G.J., Rosquin, I.J., Denys, P.N., Evans, I.J., Lathouwers, J.E., Lefèbvre, M.P., Dunwell, J.M., van Tunen, A., Krens, F.A., 1996. A high efficiency technique for the generation of transgenic sugar beets from stomatal guard cells. Nature Biotech. 14, 1133–1138.

Herrera-Estrella, L., DeGreve, H., van Montagu, M., Schell, J., 1983. Expression of chimaeric genes transferred into plant cells using a Ti plasmid derived vector. Nature 303, 209–213.

Kallerhoff, J., Perez, P., Bouzoubaa, S., Ben Tahar, S., Perret, J., 1990. Beet necrotic yellow vein virus coat protein-mediated protection in sugar beet (*Beta vulgaris* L.) protoplasts. Plant Cell Rep. 9, 224–228.

Kieser, T., 1984. Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12, 19–36.

Piatak, M., Luk, K.-C., Williams, B., Lifson, J.D., 1993. Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species. BioTechniques 14, 70–79.

Richards, K.E., Tamada, T., 1992. Mapping functions on the multipartite genome of beet necrotic yellow vein virus. Annu. Rev. Phytopathol. 30, 291–313.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Scadden, D.T., Wang, Z., Groopman, J.E., 1992. Quantitation of plasma human immunodeficiency virus type 1 RNA by competitive polymerase chain reaction. J. Infect. Dis. 165, 1119–1123.

Smith-White, B.J., Preiss, J., 1992. Comparison of proteins of ADP-glucose pyrophosphorylase from diverse sources. J. Mol. Evol. 34, 449–464.

Yanisch-Perron, C., Vieira, J., Messing, J., 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC vectors. Gene 33, 103–119.

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DNA and Protein Analysis throughout the Industrial Refining Process of Sugar Cane

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Abstract

The amount, nature, and fate of DNA and protein in the major purification fractions generated during industrial scale refining of sugar cane into raw sugar by the diffuser and tandem roller mills was determined. The presence and size of sugar cane DNA were estimated using PCR and sugar cane specific primers that amplified fragments of various sizes from different segments of the repetitive intergenic region (IGS) of the 25S rDNA. Both the maximum fragment size capable of amplification and the amount of DNA decreased as refining progressed, indicating sequential degradation during the milling. However, PCR still detected minute quantities of sugar cane DNA in raw sugar (<10e-3 ppm). Using a bicinchoninic acid assay on trichloroacetic acid precipitated sodium dodecylsulfate-extracts, protein was found in all mill fractions and decreased from 4500 to 10 ppm as sugar cane was refined to raw sugar. Analysis of these extracts by one- and two-dimensional gel electrophoresis suggested a gradual degradation of proteins during refining. Shotgun proteomic analyses identified complex populations of sugar cane proteins, or peptides thereof, in all mill fractions, but the population complexity decreased during processing. Retail-purchased refined cane sugar showed no detectable protein or DNA (<2 ppm protein; 0.001 ppm DNA).

Keywords: *Sugarcane, Refining Fractions, DNA, Protein, Detection, Proteomics*

Introduction

Crystalline sugar is manufactured from sugar cane by a multistep extraction and purification procedure using either a tandem roller mill or diffuser mill (Figure 1). Cut cane pieces (CP) are first shredded, imbibed with water and then crushed between sets of rollers to release the primary juice (PJ) (tandem mill); alternatively shredded cane is extensively lixiviated (rinsed and percolated with recycling ~80°C water) to obtain the primary juice (diffuser mill). Cane residue (bagasse, BG) is dried and used as boiler fuel, animal feed, or in paper manufacture. Primary juice is filtered and then clarified by heating in the presence of lime to precipitate impurities, especially protein. After filtration to remove the precipitate called filter cake (FC), the clarified juice (CJ) is concentrated by evaporation resulting in precipitation of raw sugar crystals (RW). The evaporation/crystallization process is repeated until no more sucrose crystallizes.

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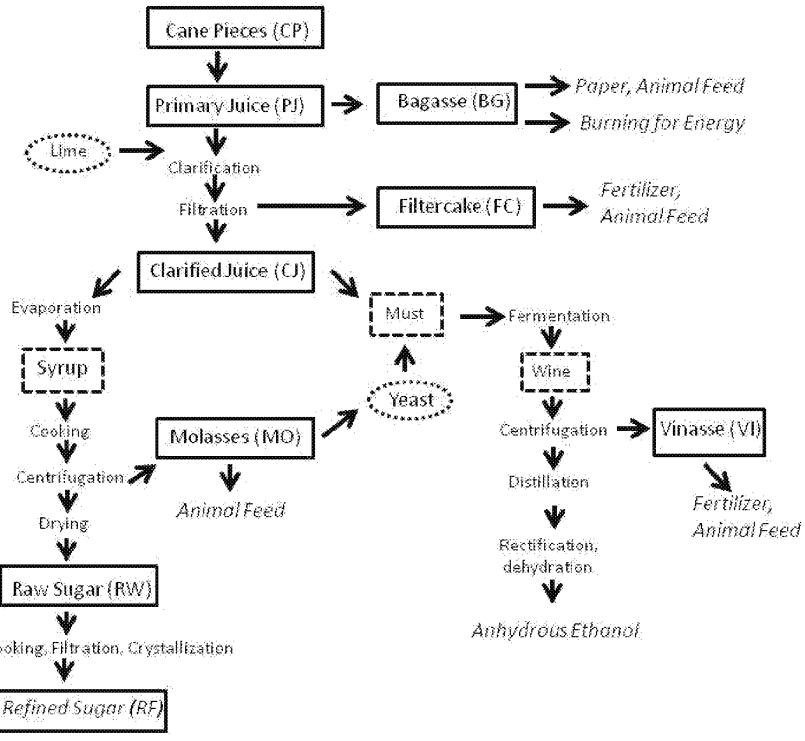

Figure 1: Processing of sugar cane into various end products. Major mill fractions analyzed in this work are in solid boxes. End products are italicized; intermediate products are in dashed boxes. Exogenous additions to fractions are in ellipses.

The residual liquid called molasses (MO) is mixed with yeast and fermented to produce ethanol. After recovery of the ethanol the residual fermentation solids are removed by centrifugation to yield vinasse (VI) which can be used as fertilizer or animal feed.

The objective of this study was to determine the fate and concentrations of sugar cane DNA and proteins throughout the industrial refining process of sampling eight different fractions (Figure 1). The fate of nucleic acid and proteins during sugar manufacture from sugar beet and sugar cane has been published (Klein et al., 1998; Oguchi et al., 2009; Taylor et al., 1999; Joyce et al., 2013; Potter et al., 1990). These reports focused on the detection of specific fragments of the foreign DNA present in the transgenic plants used as starting materials. Here we report the development of robust and highly sensitive methods that enable detection and sizing of the total DNA and protein of conventional sugar cane throughout the refining process. Methods were tested on eight mill fractions from two commercial sugar cane mills in Brazil, one using the newer diffuser extraction process and one using the more traditional tandem roller mill process. The methods were shown to be rapid and very sensitive, routinely capable of detecting protein and DNA in very concentrated, dirty, and complex samples above the 5 and 0.001 ppm levels, respectively. Results showed DNA and protein are present in all mill fractions; however the amount and size significantly decrease as the cane was refined to raw sugar.

Materials and Methods

Sample Acquisition and Preliminary Processing

Samples were collected at two different industrial sugar cane mills in Brazil: a diffuser type ("mill V") and a tandem roller type ("mill G"). Eight sample types were collected in triplicate from each mill: CP, BG, FC, PJ, CJ, MO, VI and RW (Figure

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1). CP samples were collected in plastic bags and immediately placed on dry ice. All other samples were collected in polypropylene bottles and placed immediately on dry ice. Samples were then stored at -80°C until shipment on dry ice to the US. Upon arrival CP, BG, and FC samples were lyophilized for four to five days, resulting in a moisture content of <5%. FC samples were placed at -80°C, while CP and BG samples were ground to a homogeneous powder in a GM200 Grindomix (Retsch) before storage at -80°C. CJ, PJ, MO, and VI samples were not processed before analysis. Sugar cane leaf samples were harvested from greenhouse-grown plants (varieties CP-89-2143 and SP-70-1143), placed immediately on dry ice, and then ground to a homogeneous powder as above before lyophilization for three days and stored at -80°C.

DNA Isolation

DNA was isolated from samples using Plant DNA miniprep or maxiprep kits (Qiagen) as per the manufacturer's instructions except where noted. When the miniprep kit was used, 100 mg samples were ground in a mortar and pestle with 400 µL of extraction buffer, except for the BG and FC samples, where 1200 µL of buffers were used and volumes of all other reagents were increased proportionally. When the maxiprep kit was used 1g of material was extracted. The final elution volume was 200 µL for the miniprep extractions and 1 ml for the maxiprep extractions. A 600 µL aliquot from each maxiprep extraction was then reduced to 20 µL by vacuum reduction at room temperature. DNA isolations were independently performed from each mill fraction at least three times.

Total DNA Quantitation

DNA concentrations were determined using both spectrophotometric and flurometric methods. Purified DNA samples were measured at 280 nm, 260 nm, and 230 nm (NanoDrop 8000; ThermoFisher) and in an Applied Biosystems Step-One qPCR system using SYBR green. DNA samples from each extract were also analyzed on 1% agarose gels with TBE buffer and visualized under ultraviolet light (UV) following ethidium bromide staining.

PCR Primers

The intergenic spacer region of the sugar cane rDNA repeat unit was amplified using a pair of primers designed from the sequence of the conserved 18S and 25S coding regions (25SCONS and 18SCONS; Table 1) and purified sugar cane DNA as the target. The amplicon was purified by agarose gel electrophoresis and sequenced (Supplementary Figure 1S). The sequence was analyzed using BLASTN at NCBI and the region that was specific to sugar cane and other related monocots (e.g. *Sorghum bicolor, Zea mays*) identified. Primers were designed to specifically amplify fragments of different sizes from the sugar cane intergenic spacer region (IGS; Figure 2).

The sequences of the primers are given in Table 1. The primer pairs and the expected fragment sizes are: 4L/4R – 273 bp, 4L/5R – 552 bp, 7L/7R – 800 bp, 2L/3R – 1760 bp.

Table 1

Primer Sequences

Primer Name	Primer Sequence (5' – 3')
25SCONS	AAAACACCAGCTCCCCAAC
18SCONS	AAGGCAACACGTAACCGAAC
Cane2L	AGTGAAATCACCGGGCATC
Cane3L	GGTTACGTGTTGCCTTCGTC
Cane4L	AAAAGCACAAGCGAGGTGTC
Cane4R	TTTGGACCTGTGACTGCTTG
Cane5R	GTTGGGATTGTTTCGGTGAC
Cane7L	ACCAAAAGCACAAGCGAGGT
Cane7R	GCCTAGCTGGGAGGGCTA

PCR Method

PCR reactions were performed in 25 µL with variable amounts of template, up to a maximum of 50 ng per reaction with primer concentrations of 0.2 µM. When DNA concentrations were lower than 5ng/ µL, 10 µL of the DNA preparation was used. The amplification program used an initial denaturation step at 98°C for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min. Two different polymerases, GoTaq (Promega) and Speedstar (Clontech) (1.25 units each) were added to ensure that fragments of different sizes could be amplified. Following amplification, PCR products were visualized on 1.5% agarose gels run in 0.5X TBE buffer, stained with ethidium bromide, and photographed under UV.

Sugar cane sequence of amplified bands from conserved region of 25S ribosomal RNA into the intergenic spacer region.

GTGGCGCGAAGCTACCGTGTGCCGGATTATGACTGAACGCCTCTAAGTCAGAATCCAAGCTAGCAACCGGCGCCTGTGCC
CGCCGCTCGCCCCGACCCACATTAGGGCGTTCGCGCCCCAAGGGCCCGTGCCACTGGCTCAGCCCGCCCGGCCGACGCG
CCGCGGCGGGCCCCCTCGAAGCTCCCTTCCCAACGGGCGGCGGGCTGAATCCTTTGCAGACGACTTAAAACGCGACGGG
GCATTGTAAGTGGCAGAGTGGCCTTGCTGCCACGATCCACTGAGATCCAGCCCTGCGTCGCACGGATTCCTCCCTCCCCCC
CTCCACGCCCCCGGGCCGAGTCAGGCTCCCACCCCGCGTGGCCGTGGCCGACCCTGCCGAACGGCACCGCGCTCGTCTC
CGGCCAAAGGCAAGGCAGCCCANAAATGAAATTGGCTAAGTGAAATCACCGGGCATCCGCTCCGTGCGCCCTGGGCAAAA
ACACCAGCTCCCCAACGCGTCCGGGGCCAAAAACACCAGGTC
GGCCACATGGTCCGTGAGCANCCCAAGTCCCCGGAAAGCCGGCTTTCCTACACGTGACAGATATGCGCCCGGGGCAGTTC
TCCCANGCGGCANGGCTGAAAACCNNCGGTTCTCCCANGCGCCNGAGCTGAGACACANCGNTNNNCGGACTGGCCAANTC
CCCCNGGCCGACGGCACCCGGTGGGGGGAAACANCGGTTCTCCCTGGCGCCCGGGCCNAAAACCANNAGTTCTCNCANG
NNNTCTNCGGAAGACAGGNCAAGTCCCCGGGCCGACTGCACCCACGGGGCGNAAACAGCGGGNTCNCCCTGGCGCCANG
GCCNAAACCNNNNTTNGCCCANGCGCCGGCGCTGAGACNCANCGNTTCGGCAGGNGGTNCNCNGAAGACAGNCNAGTCC
CCGGGNCGANNNCGCCNCGGGGNGGAAACAGCTGTTCNNCNTGGCGNCAGGGCTGAAACAGCAGT

Sugar cane sequence of amplified bands from conserved region of 18S ribosomal RNA into the intergenic spacer region.

GATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTGGATGTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAAT
TCTCCGTCACCCGTCACCACCATGGTAGGCCCCTATCCTACCATCGAAAGTTGATAGGGCAGAAATTTGAATGATGCGTCGC
CGGCACGAGGGCCGTGCGATCCGTCAAGTTATCATGAATCATCGGATCAGCGAGCAGAGCCCGCGTCAGCCTTTTATCTAA
TAAATGCGCCCCTCCCGGAAGTCGGGGTTTGTTGCACGTATTAGCTCTAGAATTACTACGGTTATCCGAGTAGCACGTACCA
TCAAACAAACTATAACTGATTTAATGAGCCATTCGCAGTTTCACAGTTCGAATTAGTTCATACTTGCACATGCATGGCTTAATC
TTTGAGACAAGCATATGACTACTGGCAGGATCAACCAGGTAGCACGTCCTCGCCGACGAGCCGGCGCCGGCCTCCGCACG
AGGCATCGGCCGGGCTGGCTGTCGTTGTTTCCAAACGGACTCGGTTTCTAGGCACGGGAGGCCAACGCCCTCCGGCCTTC
GGCATCAGCCGCATCCGCAACCAAAAGCACAAGCGAGGTGTCCTTGGCGGTGCCGCCCTAGGAGGACGACATCACGAGGC
AACGCCGCAAGTGCTCTAGAGCCGACGAGCCACACCCAGAGGGTGCGCTCGACGAAGGCAACACGTAACCGAACGCAACT
TCCCGTGGGACAGGTAGCAGCACGCAAGCACTCGTCAACGCAGCAGGCAAAAGGTGCCCGCACGAATGAAGGGACGCTG
GCGCTAGGAGTCAGCCGCACGCCGGCGGGGGTCCTCCAAGCAGTCACAGGTCCAAAACAACTCATGCGCCAGCGTAGCCA
CGACGGTTAAGCCATCCAAAGCATCCCTCCGCGCTAGGCACGGTGAGTCTGCTTGCGAGGACGGCAACCGAGGGTCCACA
GAGCGCGAGAGAAACCGAAACTTATCCAACAGCGGGCCAAAACCGTGCAGCCGAAACCGTTCCAGCAACAAGATTCCTCCC
ATGGGAGCCAGCTGCCCGCAGCAGCCCGCAGAAGCAGGCCACCACAGGCTGCCGGCCGTCCAAGAAGGCCGCGAGTCAC
CGAAACAATCCCAACAACACGTTGGCCACCCCAGCCAGCCAGCTACCCACAGCATCCCGCCAAAGCAGGCCGCCGCAGGC
TTCCGCCCGGCCAAGAAGGCGGCGCATCATCAAAACAGTTCCAACAACAAGCTAGCCACCCTAGGCAGCCAGCTGCCCAAA
GCAGCCCGCCGAAGCAGGCCACCGCAGGCTGCCGACCGTCCAAGGAGGCCGCAAGTCACCGAATCCGTTCCAACAACAC
GCTAGCCCTCCCAGCTAGGCAGTCGCCCGCAGCAGCCCACCCAAGCAGGCCGCCGTAGGCAGCCGGCCACCCNGGAGGC
CGGGAGTCGTTCCGACACCACGCTGGCCACCGAGCCAGCCCGCGGCCCATAGCAGCCCG

Sequence of the fragment amplified from raw sugar using primers 4L and 4R

AAAAGCACAAGCGAGGTGTCCTTGGCGGTGCCACCCTAGGAGGACGACATCACGAGGCAACGCCGCAAGTGCTCTAGAGC
CGACGAGCCACACCCAGAGGGTGCGCTCGACGAAGGCAACACGTAACCGAACGCAACTTCCCGTGGGACAGGTAGCAGCA
CGCAAGCACTCGTCAACGCAGCAGGCAAAAGGTGCCCGCACGAATGAAGGGACGCAGGCGCTAGGATTCAGCCGCACGC
CGGCGGGGGTCCTCCAAGCAGTCACAGGTCCAAA

Supplementary Figure 1S: Single pass sequences of PCR amplified fragments of the sugar cane large ribosomal RNA intergenic spacer regions used for designing primers:

Protein Extraction Method

Four mL of 3% (w/v) solutions of CP, BG, FC and VI were prepared in 15-mL polypropylene tubes (Sarstedt) and adjusted to 1% sodium dodecyl-sulfate (SDS) +10 mm dithiothreitol (DTT) + 10 mm Tris-HCl pH 7.5 (SDS-extraction buffer, SEB). Similarly 15-20% solutions of PJ, CJ, MO, RW, or retail-purchased refined sugar (RF) were prepared and identically adjusted. All samples were the heated at 65°C for 60 min with occasional mixing by inversion. Tubes were centrifuged for 15 min at 25°C and 6500 x *g* in a swinging bucket rotor. CJ-containing samples were not centrifuged as some SDS precipitated at 25°C but not 65°C. Three ml of 1% sodium deoxycholate (DOC) was mixed into 2mL of each supernatant followed by 1.25 ml of 50% trichloroacetic acid (TCA). After mixing well and sitting 15 min on ice, the tubes

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were centrifuged at 6500 x g for 15 min at 7°C and drained for 5 min. Then, 1.5 ml of acetone was added, the tubes were vortexed for 15 sec, and incubated 25°C for 15 min with an occasional vortexing. If necessary the tubes were sonicated for 1 min in a Branson 1510. Samples were placed on ice for 10 min, centrifuged, supernatants removed, and the tubes drained. Then 1.5 ml of 85% acetone was added and the tubes were vortexed, centrifuged, drained and dried at 37°C for 15 min. The precipitate was dissolved in 0.2-0.5 ml of 0.5 % SDS + 10 mm Tris-HCl 7.5 at 65°C for 20 min with occasional vortexing and/or sonication. For preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins extracted from CJ, MO, RW and RF the procedure was scaled up seven-fold. Protein content was determined using the microplate Micro BCA protein assay (Thermo Fisher) as recommended by the manufacturer. Protein at concentrations as low as 4-8 µg/ml of mill fraction could be detected with this protocol. The Overall recovery of protein from fractions with low protein concentrations (CJ, MO, RW, RF) was >50% as determined by analyzing mill fractions spiked with known amounts of protein added (7 µg /ml each of bovine serum albumin and lysozyme).

In some preliminary experiments, proteins in mill samples were extracted with LBT (7 M urea + 2 M thiourea + 12 mm Tris-HCl 7.5 + 4% CHAPS + 12 mm DTT) (Amalraj et al., 2010), precipitated with DOC/TCA, washed and redissolved in the same fashion as when using SEB. A P-PER kit (Pierce) was also used for additional preliminary extractions, according to manufacturer's instruction.

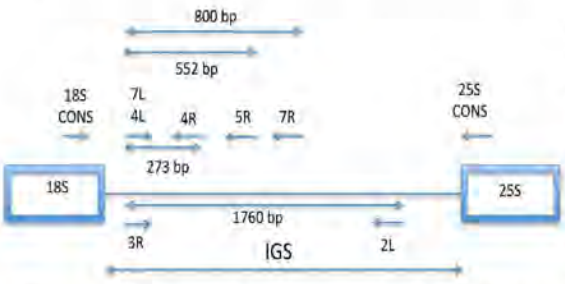

Figure 2: Position of primers within the rDNA repeat unit. 25S CONS and 18S CONS are conserved primers within the 25S and 18S coding regions, respectively. The remaining primers are within the Intergenic Spacer Region (IGS).

Gel Electrophoresis

Analytical and preparative SDS-PAGE was performed using 4-20% gradient gels (Thermo Scientific 0025244). For analytical-scale samples, 10-60 µg of protein in 15 µL, were adjusted to 2% SDS + 2% beta-mercaptoethanol + 15% glycerol + 0.05% bromophenol blue and boiled for 5 min before loading. The gels were run at 50V, treated with BioSafe Coomassie Stain as per manufacturer's instructions and photographed with a digital camera. Peptide mass fingerprinting to identify single protein bands excised from analytical gels was performed essentially as described by Schell et al. (2011).

Preparative SDS-PAGE was run analogously, except 150 µg of protein was loaded on the gel; for RW, 35 µg was loaded. Electrophoresis was carried out until the bromophenol blue dye had migrated one-third of the way down the gel. The stained portion of each lane was excised and cut in half producing a high-molecular mass containing a slice (50-250 kDa) and low-molecular mass (6-50 kDa) containing slice.

Samples for two dimensional gel electrophoresis (2-DGE) were prepared by SEB extraction and DOC/TCA precipitation as described above. Samples were processed using a ReadyPrep 2D Cleanup Kit (BioRad) followed by dissolving in Ready-Prep Rehydration Buffer (BioRad). Samples containing 100 µg of protein were applied to 11 cm pH 4-7 Ready-Strip IPG Strips (BioRad) for 12 hr. IEF was performed using the BioRad Protean IEF cell at 20°C with the following program: 250V with a linear slope for 20 min; 8000V with linear slope for 150 min; and 8000V with a rapid ramp for 20000 V-hr). IEF the strips were processed as recommended by the manufacturer. The strips were applied to 1.0 mm Criterion precast 12.5% gel (BioRad) and run at 200 V for 1 h. Spectra Multicolor Broad Range molecular mass standards (Thermo Scientific) were run adjacent to each strip. Gels were treated with BioSafe Coomassie stain as above and then scanned using the Amersham BioSciences image scanner and the associated 2D Platinum software used to view, analyze, and compare the images. Spots were identified using intensity >4000, area >2, volume >6000, and saliency >200.

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Mass Spectrometry

Shotgun proteomic analyses (liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)) were performed on a Thermo-Fisher LTQ Orbitrap Elite Mass Spectrometer coupled with a Proxeon Easy NanoLC system. Tryptic peptides were derived from the high and low molecular mass preparative SDS-PAGE gel slices essentially as described by Schell et al. (2011), except reagent volumes were increased 3 fold. Extracted peptides (~ 0.25 µg) were first passed through a PepMap 5 mm x 300 µm precolumn (Dionex). The eluate was loaded on a self-packed 12 cm x 100 µm column/emitter containing 200 Å 5 µM Bruker MagicAQ C18; peptides were eluted with a 500 µL gradient of solution A (0.1% formic acid in acetonitrile) and solution B (0.1% formic acid). The gradient started with 5% B, increased to 40% B in 40 min, increased to 60% B in 15 min, and increased to 95% B in 10 min. The Top 10 data-dependent acquisition method was used to acquire data. Proteome Discoverer 1.3 (Thermo-Fisher) software was used by Mascot database search program (Matrix Science) for protein identifications. Databases searched were:1) a set of 121,000 expressed sequence tags (ESTs) from sugar cane (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=s_officinarum) translated in all six possible reading frames); 2) a *Sorghum bicolor* proteome predicted from its complete genome sequence (http://www.ncbi.nlm.nih.gov/genome/108); and 3) the NCBInr protein database of all nonredundant predicted protein sequences. We defined positive protein identifications as those which had Mascot scores of >70 based on data from at least 2 unique peptides; at this stringency identifications are at the >99% confidence level.

Results

Partial Sequence of the Sugar Cane Intergenic Spacer Region

Primer pair 25SCONS/18SCONS was designed from conserved regions of the plant 25S and 18S ribosomal RNA genes, and used to amplify purified sugar cane DNA isolated from sugar cane leaves. The amplified fragments were gel-purified, sequenced, and then primers specific to the sugar cane intergenic spacer region (IGS) near either the18S or 25S coding regions were designed. The primers were tested on sugar cane leaf DNA, and in all cases the expected fragment size was observed. The new primers 2L and 3R were used with sugar cane DNA and the resulting fragment isolated and sequenced. This sequence was used to design additional primers that amplified a range of fragments of different sizes from the IGS. The primer sequences are in Table 1. The positions of the various primers and the sizes of the fragments amplified are shown in Figure 2.

DNA Quantitation

The quantity of DNA isolated from the various mill fractions was determined by three different methods. The amounts measured by UV spectroscopy decreased from 1.3 ppm in CP to 0.3 ppm in PJ, while BG and FC contained amounts similar to those found in CP (Table 2). The amount of DNA in the subsequent processing fractions CJ, MO and RW was below the limit of detection even when using the most sensitive (SYBR green) method. Using agarose gel electrophoresis, DNA was detected only in the CP, BG and FC fractions (Figure 3). The DNA from the BG and FC fractions was degraded into much smaller fragments than the DNA from the CP, an observation that was supported by the amplification products observed in PCR reactions discussed below.

Table 2

Amount of DNA in Processing Fractions of Mills G and V

Sample	DNA in Eluate ng/µL	Total µg DNA Recovered per g Starting Material (ppm)
PJ-G	0.003 ± .002	0.3 ± 0.2
PJ-V	0.003 ± .003	0.3 ± 0.3
FC-G	11 ± 4	1.1 ± 0.4
FC-V	14 ± 3	1.4 ± 0.3
BG-G	10 ± 3	1.0 ± 0.3
BG-V	15 ± 4	1.5 ± 0.4
CP-G	11 ± 5	1.1 ± 0.5
CP-V	13 ± 5	1.3 ± 0.5

For samples CJ, MO, VI, and RW from both mills, as well as retail-purchased refined sugar, DNA was below the limit of detection with SYBR green: < 0.002 pg/µl; < 0.4 ng/g; 0.0004 ppm.

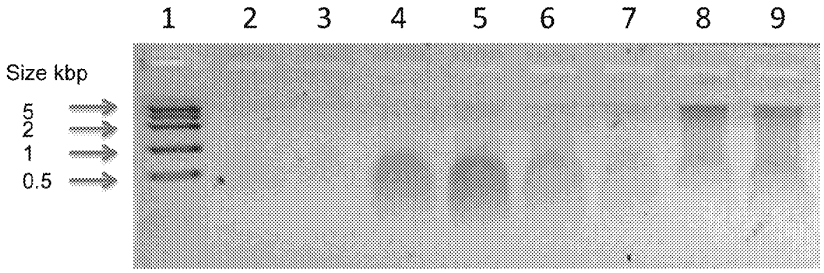

Figure 3: Agarose gel electrophoresis of DNA extracted from mill processing fractions. 15µL of DNA extract was loaded. Lanes: 1, molecular weight marker II; 2, PJ-G; 3, PJ-V; 4, FC-G; 5, FC-V; 6, BG-G; 7, BG-V; 8, CP-G; 9, CP-V.

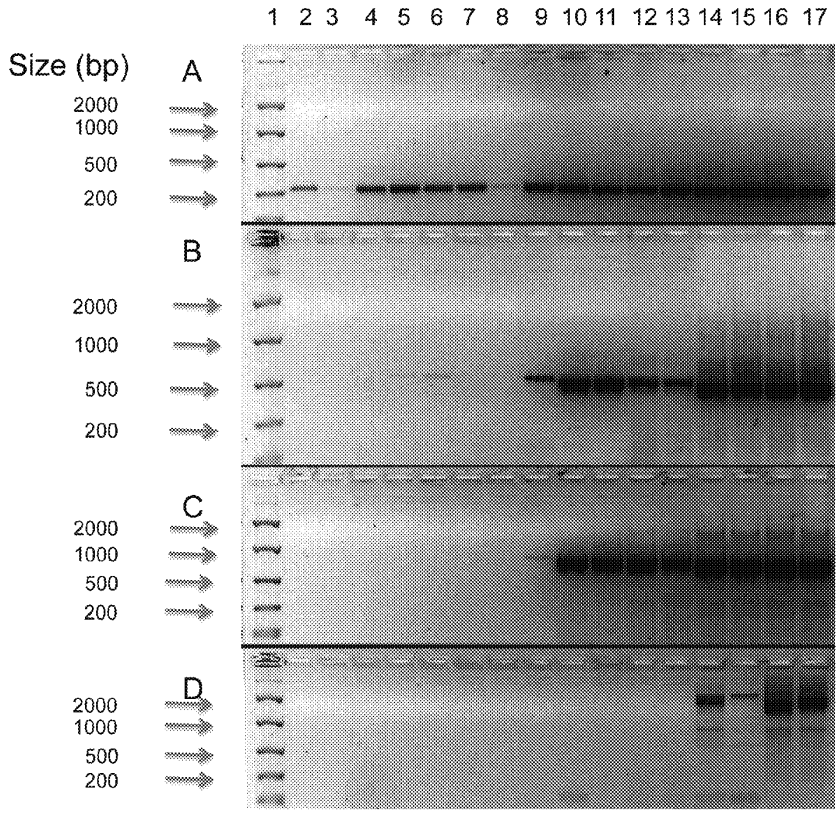

Figure 4: PCR amplifications of DNA extracted from mill processing fractions. Lanes on the 1.5% agarose gel show a representative PCR reaction from each fraction from mill G and mill V. Lanes: 1, molecular weight marker I; 2, RW-G; 3, RW-V; 4, MO-G; 5, MO-V; 6, VI-G; 7, VI-V; 8, CJ-G; 9, CJ-V; 10, PJ-G; 11, PJ-V; 12, FC-G; 13, FC-V; 14, BG-G; 15, BG-V; 16, CP-V; 17, CP-V. Panel A: Primers 4L/4R; Panel B: Primers 4L/5R; Panel C; Primers 7L/7R; Panel D: Primers 2L/3R.

Detection of Sugar Cane DNA Sequences in Extracts by PCR

For the determination of the presence of sugar cane DNA in the mill fractions, PCR amplifications with a series of primer pairs designed from the repetitive IGS region of the large ribosomal RNA genes were performed (Figure 4). Using primer combinations that amplified different sized

fragments, the maximum size of DNA remaining in each fraction was estimated. The primer pair 4L/4R amplified the shortest fragment (273 bp) while the pair 2L/3R amplified the largest fragment (1760 bp) (Figure 2). PCR products of the expected size were amplified from the DNA preparations from all of the mill fractions when using the primer pair 4L/4R, although in very different final amounts (Figure 4; Panel A).

These data are from endpoint PCR with different amounts of input DNA. Therefore the intensities of the bands are not directly reflective of the actual differences in DNA in the reaction since those reactions with more input DNA reach saturation at a lower number of cycles. More realistic comparisons can be made by altering the amplifycation cycle number. Amplification from the FC, BG and CP was observed even when the number of PCR cycles was reduced from 35 to 20. Therefore the amplification differences apparent in Figure 4 represent a more than 10000-fold difference in target DNA. The amplicons from RW, VI, MO and CJ using 4L and 4R were gel-purified, reamplified, and sequenced. The sequence matched that of the previously determined sugar cane IGS region, confirming the presence of sugar cane DNA in these fractions (Figure. 1 and Supplementary Figure 1S). In all of the presented data amplifycations from only one of the samples is shown. However, DNA samples from all of the fractions were tested using all of the primers, including all the 6 replicate extractions for RW, VI, MO and CJ which gave results similar to those presented.

To test for possible contamination during the extraction process, an equivalent volume of double distilled water was substituted for the RW mill fraction and simultaneously extracted using the same reagents. These water extractions showed no amplification with any of the primers. Therefore, all the mill fractions contain sugar cane DNA with a size of >273 bp. As the PCR-target size increases from 273 bp to 1760 bp, the amplification from the samples from the later stages of the processing chain is reduced (Figure 4; Panel B-D). None of the DNA preparations from RW supported amplification of fragments as large as 550 bp. VI or MO did not support amplification of 800-bp long fragments and only one of the CJ derived samples gave detectable amplification of an 800-bp product (Figure 4, panel C). Finally, only DNA preparations from the BG and CP directed amplification of the 1760-bp fragment. These data indicate progressive degradation of sugar cane DNA into smaller pieces during the refining process. These data are also consistent with previous studies, which failed to identify fragments >700 bp in the downstream sugar refining fractions (Joyce et al., 2013).

To estimate the amounts of sugar cane DNA in mill fractions with low DNA, we performed endpoint PCR comparing the amount of amplified product obtained using the 4L/4R primer pair and serial dilutions of the DNA from CP and the concentrated DNA samples from other fractions. DNA extraction samples from RW, VI, MO and CJ were estimated to contain less than 1 pg DNA per g of starting fraction. This is approximately equal to the DNA from a single cell per 4 g of fraction, using 4 pg as the value for the 1C nuclear DNA content of sugar cane (Kew Plant DNA C-values, data.kew.org/cvalues/).

Measurement of Protein in Sugar Cane Mill Fractions

Our next goal was to develop robust and sensitive methods to extract and characterize the protein content of sequentially derived processing fractions from the two mill types. A preliminary evaluation was made of the performance of two plant protein extraction methods: 1) 60 min at 65°C in LBT; 2) the commercial P-PER Plant Protein Extraction Kit (Pierce); and one general protein extraction method: 60 min at 65°C in SEB. Initial extracts required subsequent concentration/purification by precipitation with DOC + TCA before protein measurement since most of the downstream mill fractions (CJ, MO, RW, and RF) have a low protein content relative to the amount of DTT and dissolved solids which were likely to interfere with the protein assay.

In general, SEB extraction yielded 20 to 100% more protein from most mill fractions compared to LBT; with the exception of VI, the P-PER kit yielded 10 to 50% less protein than SEB extractions. The limit of protein detection after LBT extraction was 3 fold higher than for SEB extractions, in part due to the dilution of the sample caused by the addition of large amounts of urea/thiourea in LBT to the aqueous samples. Some LBT extracts gave poor results during SDS-PAGE analysis (not shown). Given these limita-

tions and fact that LBT and P-PER are optimized for extraction of protein from plant tissues (only two of eight mill fractions are "plant tissues"), the SEB extraction method was chosen for all subsequent analyses. Using our SEB extraction and TCA precipitation method of water or 20% refined sugar samples containing 1 to 100 µg per ml of a protein standard indicated the lower limit of detection as 5 µg/ml protein.

Two independently collected samples of mill fractions were extracted with SEB, precipitated with TCA/DOC, dissolved in 0.5 x SEB without DTT and assayed for protein content with a BCA-based assay (Table 3). For the most part variation between values obtained for each fraction from either mill type was not large (<30%). The protein content of CP was found to range between 3700 and 4600 ppm, consistent with the range of 2000-5000 ppm reported by Amalraj et al. (2010) for the 5 extraction methods they tested on experimental farm grown sugar cane stalks and the 20000 ppm average reported for dried cane stalks (feedipedia. org/node/14465). The first mill products, BG and PJ, each contain about half of the protein from the shredded cane stalk starting material (assuming 50% water imbibition). During clarification of the PJ fraction >95% of the protein is removed by the majority (>75%) ending up in the FC. During further processing of the CJ, the majority of protein in this fraction (~35 µg/ml protein; 200 ppm) ends up in the MO with only a small part (4%) remaining in the RW. We estimate that there is <1 g of protein in the RW derived from one metric ton of cane; this amount represents 0.005 - 0.020% of the original protein in the CP.

Since the protein levels detected in RW were so low, a protease digestion experiment was performed to ensure that the BCA reactive material is indeed protein. Digestion of 20 µg of RW-derived protein with 2 µg each of pronase and proteinase K reduced the intensity of the BCA reaction by 75%; an analogous treatment of 20 µg of bovine serum albumin also caused a 75% reduction. Thus, we are confident in our detection value of 9 to 15 ppm protein in raw sugar. It is consistent with the previous estimate of 20-60 ppm reported by Goodshall and Roberts (1976).

We also analyzed 3 batches of locally-purchased refined sugar. The average protein content was 1.4 ppm. However, this is close to the limit of detection as SEB buffer controls gave an equivalent value of 0.5 ppm. Furthermore SDS-PAGE analysis of 10 µg of 'protein' extracted from refined sugar by SEB showed no protein as indicated by Coomassie Blue staining (see below). Thus, we could not reliably detect protein in refined sugar; if present, the level of protein is probably well below the 1 ppm detection limit of our assay.

Table 3

Protein Content in PPM of Mill Fractions from Sugarcane Processing

	Mill V (diffuser)		Mill G (tandem roller)		
Mill Fraction	**Sampling 1**	**Sampling 2**	**Sampling 1**	**Sampling 2**	**% Dry matter Mill V/MillG**
CP	3840 ± 330	4150 ± 1060	4620 ± 420	3700 ± 960	100/100
BG	3300 ± 230	2680 ± 450	2810 ± 215	3290 ± 360	100/100
PJ	3620 ± 435	3510 ± 316	3140 ± 365	4240 ± 435	21/23
CJ	244 ± 24.2	234 ± 27.2	183 ± 19.6	148 ± 15.4	18/22
FC	10400 ± 1360	13500 ± 1520	11600 ± 1870	12300 ± 3230	100/100
MO	286 ± 88	274 ± 24.7	295 ± 52	254 ± 32	87/92
VI	3260 ± 195	3500 ± 225	3770 ± 237	3950 ± 375	3.1/3.2
RW	14.3 ± 4.1	8.6 ± 2.5	14.7 ± 4.6	13.3 ± 3.9	100/100

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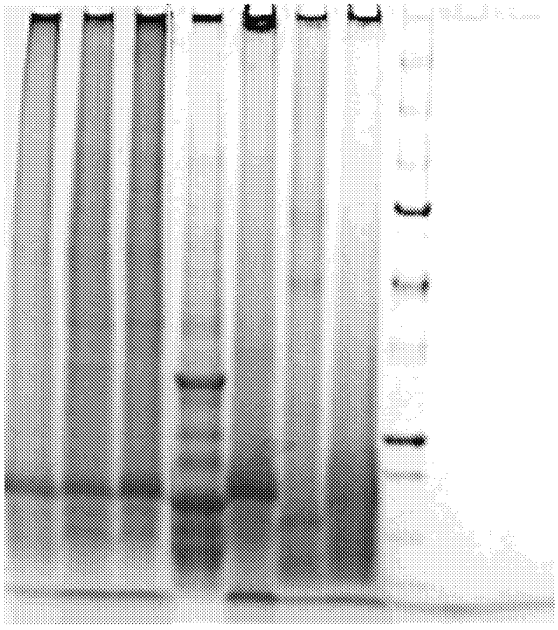

Figure 5: SDS-PAGE gel of protein in fractions of Mill V. 60 µg of extracted protein was loaded, except RW and RF where 20 µg and 7 µg respectively were used. Std; molecular mass standards; sizes shown at right. Boxes; protein bands subjected to PMF

Identification of the Origins of Proteins or Peptides in Mill Fractions

Protein samples from each fraction were subjected to SDS-PAGE analysis. The results for fractions from mill V are shown in Figure 5. In general, proteins isolated from all fractions smeared. Bands possibly representing residual intact proteins were faintly detectable in the smeared background for CP, BG, PJ, VI, and especially CJ. MO and RW showed little or no evidence of bands or high molecular mass staining material, suggesting proteins in these fractions are fragmented; RF showed no evidence of protein. SDS-PAGE analysis of fractions of Mill G gave similar results, except for the CJ fraction where few, if any, clear bands were visible (not shown). In summary SDS-PAGE analyses of the mill fraction proteins are suggestive of a sequential degradation and/or fragmentation of proteins into smaller peptides as refining progresses and are most apparent in the MO and RW fractions.

One prominent band of ~18 kDa apparently accounting for a large proportion of stained protein in all early mill fractions (BG, CP, PJ, CJ, FC; Figure 5) was excised and subjected to peptide mass fingerprinting (PMF); results were consistent with its identification as the predicted sugar cane protein TC128535, a 20-kDa dirigent-type protein, but statistical probability was not high. However, subsequent shotgun proteomic analysis detected this protein in all mill fractions (see below). The 33-kDa prominent band in CJ was clearly identified by PMF as TC131796 which is 90% identical in amino acid sequence to a *Sorghum bicolor* protein (SORBIDRAFT_03g045490) and a 37-kDa beta-1,3-glucanase from *Zea mays* (Genbank Accession ADL60383).

Two-Dimensional Gel Analysis of Mill Fractions

Since SDS-PAGE poorly resolved polypeptides from the mill processing fractions, protein extracts from both mills were analyzed by two-dimensional gel electrophoresis (2-DGE). In contrast to SDS-PAGE, protein extracts of early fractions (CP, PJ, CJ and BG) from Mill V showed many clear and well resolved protein spots (Figure 6). However, 2-DGE analysis of later mill fractions (FC and MO) showed less than a dozen faint resolved spots; the majority of the staining material accumulated in a low molecular mass smear (<15 kDa) at the bottom of the gel (not shown). This is consistent with the SDS-PAGE results (Figure 5) and further supports the conclusion that proteins in these later fractions may be significantly degraded. 2-DGE analysis of protein extracts of fractions from Mill G were qualitatively similar to those from Mill V (not shown).

Image analysis software was used to determine the number and molecular mass of protein spots for each 2-D gel in Figure 6 and sort them into three molecular mass ranges (Figure 7). CP samples showed 417 spots, more than the highest number observed by Amalraj et al. using 5 different extraction methods and the more sensitive silver-staining detection method. PJ protein samples gave a 2-DGE pattern very similar to CP samples; the majority of the 483 spots detected were determined by the software to be the same as a corresponding protein found on the CP gels, indicating that most proteins in CP were released into the primary juice.

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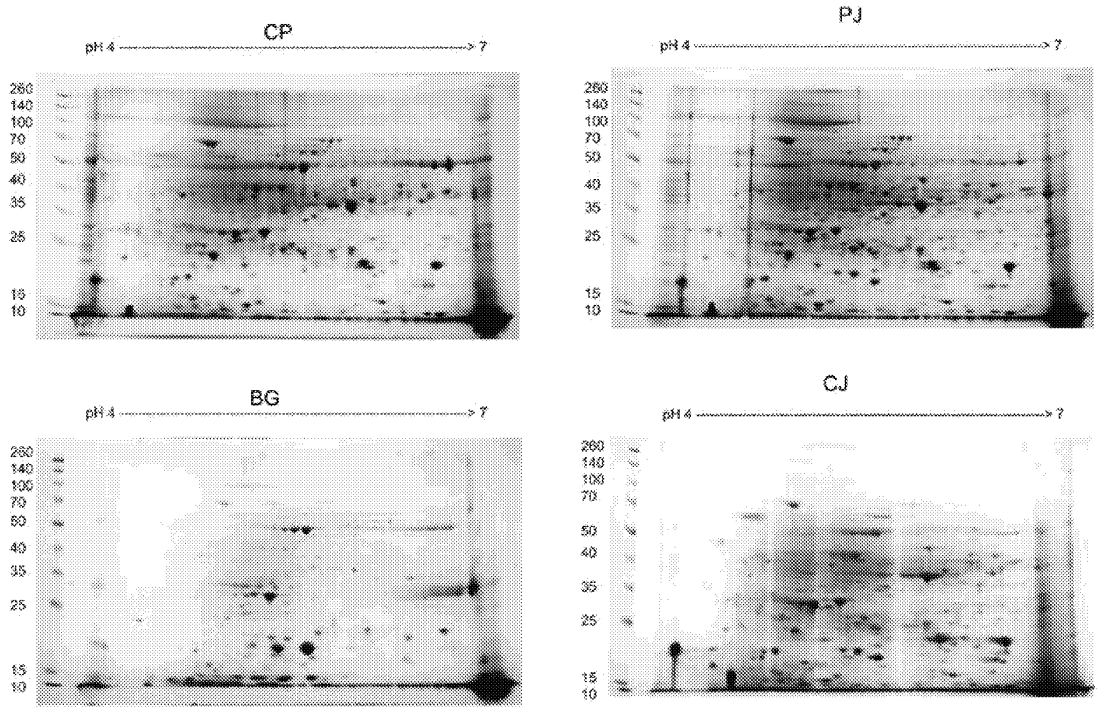

Figure 6: Representative 2-DGE for 100 µg of protein from early stage processing samples of mill V. Molecular masses of standard proteins are on the left; IEF range is at top.

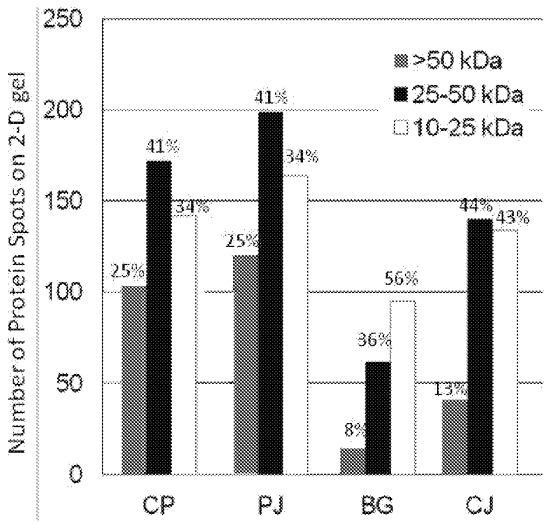

Figure 7: Analysis of protein spot number and size range for 2-DGE gels in figure 6. Numbers on top are percentages of total spots in each sample in designated molecular mass range. Total spot numbers are: CP, 417; PJ, 483; BG, 171; CJ, 315.

BG samples showed a different 2-DGE pattern and had less than half the number of protein spots observed with CP and PJ samples, suggesting BG may contain a subset of sugar cane proteins that are not efficiently extracted from the milling process. Relative to its immediate precursor fraction PJ, the total number of protein spots observed with CJ samples was 35% less with the most conspicuous loss in the higher molecular mass range. The number of protein spots >50 kDa was reduced from 120 in PJ to 45 in CJ. Less than 13% of the CJ protein spots were >50 kDa, compared to 25% for PJ.

Image analysis of the gels in Figure 6 shows that the majority of individual specific proteins present in CP and PJ are missing or reduced in CJ. This is not surprising since milling of PJ into CJ involves heating with lime which precipitates >90% of the protein into the FC (Table 3). All gels were loaded with equivalent amounts of protein to enable visualization of spots. If loading had been based on equal volumes of the fractions, some gels would have far fewer visible spots. These results demon-

strate that the milling process clearly reduces the complexity of the protein population.

To determine the identity of the proteins and/or peptides in each mill fraction, we used shotgun proteomics (i.e. LC-MS/MS analysis of tryptic peptides derived *en masse* from bulk, largely unfractionated proteins). Preparative SDS-PAGE was performed on the TCA-precipitated protein from each fraction to further eliminate non-protein materials and SDS which would interfere with trypsin digestion or mass spectrometry. Tryptic peptides were derived from whole lane gel-slices (not individual bands) and subjected to LC-MS/MS. The outputs were searched against the predicted proteome databases of sugar cane and *Sorghum bicolor,* as well as NCBInr to identify parent proteins of peptides identified by MS/MS. We used the *S. bicolor* proteome database because it is the closest evolutionary relative of sugar cane with a complete genome sequence. Furthermore, a comparison of 282,000 sugar cane ESTs of the coding DNA sequences (CDSs) of ESTs from the sorghum genome showed that 70% of sugar cane ESTs matched a *Sorghum* CDS with average amino acid sequence identity of 94% and vice versa (Nishiyama et al., 2011)

Peptides from 835 unique predicted sugar cane proteins were identified with 99% confidence in protein extracts from CP from mill G; 624 were identified from the cane used by mill V (Supplementary Table 1S). Peptides from 506 sugar cane homologs of *Sorghum* proteins from mill G were identified; 388 from mill V. More than 300 of these individual proteins were detected in cane from both mills. Of the 28 proteins identified in the cane stalk by Amalraj et al. (2010) using PMF, 23 were among the 835 identified here. PJ from mill G contained proteins or peptides from 475 predicted sugar cane proteins, while the CJ derived from it contained 244.

44 and 33 sugar cane proteins were identified in the raw sugar from mills G and V, respectively; 13 of these individual proteins or peptides thereof were found in the raw sugar from both mills (Supplementary Table 1S). Similar results were obtained searching the *Sorghum* database.

Searches of LC-MS/MS output against the NCBInr protein database implied the presence of a few nonplant proteins in some fractions, mostly keratin and a few bacterial, fungal or insect proteins. One exception was the detection of a large number of bacterial proteins/peptides in PJ and BG of mill V. Although 75% of the proteins identified in PJ by searching NCBI-nr were from sugar cane-related plants, the remaining 25% were from members of the bacterial family *Leuconostocaceae*, mostly *Weissella* sp. Proteins from *Weisella* were not detected in the CP of mill V, but were found in its BG (~ 10% of the total identifications). *Weissella* are associated with sugar cane plants and often cause spoilage or souring after harvest (Bjorkroth et al., 2002). No *Weissella* proteins were detected in any fractions from mill G or downstream fractions of mill V.

The mill fraction with the most nonplant proteins was VI. Over 40% of the proteins identified in VI from either mill by searching NCBInr were from yeast (*Saccharomyces cerevisiae*); another 50% of the proteins identified in the VI from mill V were from *Lactobacillus* sp. In contrast, only 10% of the VI proteins identified in this fraction from mill G were from Lactobacilli. Nonetheless, many sugar cane proteins/peptides remained as significant components of this fermentation residue from both mills. VI from mill G contained ~three times as many residual sugar cane protein/peptides as VI from mill V. Presence of yeast and lactobacilli proteins in VI is not unexpected as VI is a residue from a yeast-based alcoholic fermentation of MO and contamination of the fermentation by *Lactobacilli* has been reported previously (Lucena et al., 2010; Gallo, 1990.)

Analysis of MO derived from the CJ of mill V showed the presence of 311 sugar cane proteins/ peptides, while that from mill V showed 197. Approximately 108 of these were found to be coincident in MO from both mills. Although most of the protein from RW appeared degraded as judged by SDS-PAGE, we still identified sugar cane proteins or peptides: 44 from RW of mill G and 60 from mill V, and as previously noted, 13 unique sugar cane proteins/peptides were coincident in raw sugar from both mills.

Comparing all LC-MS/MS data, we found six predicted sugar cane proteins/peptides present in all sequentially produced sugar cane processing fractions (CP, PJ, CJ, MO, and RW) from both mills, but only 3 that were found in all fractions from all mills (Table 4).

Supplementary Table 1S

Results of LC-MS/MS Analyses of Mill Fraction Proteins

Fraction	Database searched	Unique protein identifications from Mill G	Unique protein identifications from Mill V	Proteins identified in fractions from both mills
Cane pieces (CP)	Cane	835	624	382
Cane pieces	Sorghum	506	388	305
Cane pieces	NCBI	695	552	nd
Primary juice (PJ)	Cane	475	202	184
Primary juice	Sorghum	338	299	171
Pressed juice	NCBI	348	400	nd
Bagasse (BG)	Cane	768	348	205
Bagasse	Sorghum	497	253	213
Bagasse	NCBI	608	305	nd
Clarified juice (CJ)	Cane	244	247	46
Clarified juice	Sorghum	107	147	74
Clarified juice	NCBI	145	183	nd
Filtercake (FC)	Cane	627	266	138
Filtercake	Sorghum	355	177	148
Filtercake	NCBI	601	117	nd
Molasses (MO)	Cane	311	197	108
Molasses	Sorghum	170	109	85
Molasses	NCBI	163	111	nd
Vinasse (VI)	Cane	108	33	20
Vinasse	Sorghum	53	21	16
Vinasse	NCBI	200	202	nd
Raw sugar (RW)	Cane	44	60	13
Raw sugar	Sorghum	28	38	13
Raw sugar	NCBI	12	56	nd

nd; not done

Table 4

Sugar Cane Proteins (ESTs) with Peptides in Most or All Mill Fractions

Accession Number	Description/Annotation from Compbio
TC123310	Similar to UniRef100_A5H454 Cluster: Plasma membrane-bound peroxidase 3-2 from *Zea mays*
*CA146186	Similar to UniRef100_Q2XX98 Cluster: Pathogenesis-related protein 5 from *Zea mays*
*TC113487	Homologue to UniRef100_A2X1D2 Cluster: Phosphoglycerate kinase from *Oryza sativa*
*TC128535	Homologue to UniRef100_Q6TGM7 Cluster: Dirigent from *Saccharum* hybrid
TC135999	Similar to UniRef100_Q38769 Cluster: Permatin from *Avena sativa*
CA273807	Homologue to UniRef100_A6N0M9 Cluster: Nucleoside diphosphate kinase from *Oryza sativa*

Data is from Compbio sugar cane EST database (http://compbio.dfci.harvard.edu/cgibin/tgi/gimain.pl?gudb=s_officinarum);
*- protein found in all fractions from both mills.

These are apparently refractory to the harsh treatments that occur during processing of cane to raw sugar and/or are present in large amounts in the initial starting material. One of these TC128535 is likely the 18 kDa prominent band present in most fractions shown in Figure 5. Identifications and accession numbers of individual sugar cane proteins found in fractions from both mills and those which are shared among sequential mill processing fractions are posted online at:

https://dl.dropboxusercontent.com/u/16262713/millG_shared_proteins.xlsx

https://dl.dropboxusercontent.com/u/16262713/fc_vi_bg_millGandV.xlsx

https://dl.dropboxusercontent.com/u/16262713/millV_shared_proteins.xlsx

Conclusions

Methods were developed and applied to characterize the DNA and protein content of all major fractions produced during the milling of sugar cane into raw sugar. In contrast to previous studies, the methodology employed for DNA, particularly the use of a repetitive sequence target, and a series of primers amplifying different sized products within this target, allowed estimation of both amount and size of the DNA fragments present. Sugar cane DNA was found in all fractions from the refining process; however, in the later fractions (CJ, MO, VI, and RW) the amount was very low (<0.4 ng/g; 0.0004 ppm) and detectable only by PCR. The DNA was also of short length (<500 bp) compared to the DNA in early mill fractions. Thus, sugar cane DNA appears to be gradually degraded and lost as refining progresses; the amount of DNA in RW is >2000 fold less than in the CP starting material. PCR failed to detect any sugar cane DNA in refined sugar. These conclusions are consistent with previous studies on the detection of DNA through the refining process (Joyce et al., 2013; Klein et al., 1998; Oguchi et al., 2009; Taylor et al., 1999).

Our method developed to measure protein in mill fractions reliably detected as little as 5 µg/ml or 1 ppm of protein. We used precipitation with TCA/DOC to remove larger quantities of sugars and other contaminants in the complex and sometimes dirty sugar cane mill fractions that can cause inaccurate protein determinations. Our protein measurements were not subject to such interference since mill samples spiked with known amounts of a standard protein gave accurate values. Furthermore, protease digestion experiments confirmed that >90% of the material in the RW and MO fractions that reacted with the protein detection reagent was indeed protein.

Similar to what was observed for DNA, the concentration of protein in the sequential mill fractions decreased by three orders of magnitude as processing progressed. We estimate that the majority (90%) of the CP protein ends up equally distributed between the BG and FC from the clarification step. We also estimate that <0.02% of the initial protein in CP ends up in the 'final' RW fraction. At the limit of detection of our assay (1 ppm), we could not detect any protein in refined sugar, indicating the additional refining removes most, if not all, of the relatively miniscule amounts of sugar cane protein remaining in RW.

Results of 1-D and 2-D gel electrophoresis of the mill fractions were consistent with the progressive degradation and fragmentation of the cane protein during the refining process. Proteins in RW, FC, and MO appeared to be the most extensively degraded. To circumvent this limitation, we used shotgun proteomics to determine the origin and identity proteins and peptides present in each mill fraction. Many proteins that were reported as abundant in sugar cane stalks by Amalraj et al. (2010) were detected, as were other abundant proteins involved in plant metabolism. The number of unique proteins/peptides detected in each fraction decreased as refining progressed. This is not unexpected as most steps of the refining process are very harsh for proteins (e.g. boiling and drying); such conditions would be expected to select from a small subset of proteins or peptides in later fractions that are thermally stable and resistant to drying. The vast majority of the hundreds of proteins found in most mill fractions were from sugar cane. The three exceptions were the BG and PJ of Mill V which contained proteins of *Weissella* sp., a reported bacterial inhabitant of sugar cane and/or VI from both mills. VI contains the residual solids from a yeast-based fermentation of molasses and CJ, and thus not surprisingly contained mostly yeast proteins as well as proteins

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of lactobacilli that can contaminate large-scale alcoholic fermentations.

The methods described here may be useful in both quality control and in monitoring the fate of transgene products, both at the DNA and protein levels, in genetically-modified sugar cane or other plants and plant products.

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References

Amalraj J., Selvarai R. S., Veluswamy, G.K., Ramanujan, R.P., Muthurajan, R., Palaniyandi, M., Agrawal, G.K., Rakwal, R., and Viswanathan, R. (2010), "Sugarcane proteomics: Establishment of a protein extraction method for 2-DE in stalk tissues and initiation of sugarcane proteome reference map", *Electrophoresis* Vol. 31, pp. 1959-1974

Bjorkroth, K.J., Schillinger, U., Geisen, R., Weiss, N., Hoste, B., Holzapfel, W.H., Korkealaand, H.J., and Vandamme, P. (2002), "Taxonomic study of *Weissella confusa* and description of *Weissella cibaria* sp. nov. detected in food and clinical samples", *IJSEM*. Vol. 52, pp. 141–148

Gallo, C.R (1990), "Determinação da microbiota bacteriana de mosto e de dornas de fermentação alcoólica. PhD Thesis", University of Campinas, Campinas

Goodshall, M.A. and Roberts, M.A. (1976), "Determination of refined sugar proteins by polyacrylamide gel electrophoresis" *Proc. Tech. Session Cane Sugar Refin.Res.,* New Orleans, LA, pp. 57-72

Joyce, P.A., Dinh, S-Q., Burns, E.M. and O'Shea M.G. (2013), "Sugar from genetically modified sugar cane: Tracking transgenes, transgene products and compositional analysis", *Proc. Int. Soc. Sugar Cane Technol.",* Vol. 28, pp. 1 – 9

Klein, J., Altenbuchner, J. and Mattes, R. (1998), "Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets", *J. Biotechnology*, Vol. 60, pp. 145 – 153

Lucena, B.T.L., dos Santos, B.M., Moreira, J.L.S., Moreira, A.P.B., Nunes, A.C., Azevedo, V., Miyoshi, A., Thompson, F.L., Morais, M.A. (2010), "Diversity of lactic acid bacteria of the bioethanolprocess", *BMC Micro*., Vol. 10, pp. 29

Nishiyama, M., Vicente F., Sato P., Ferreira,S., Feltus, F. and Souza**, G**. (2011), "The *Saccharinae* transcriptome", in Paterson A.H., (Ed) *Plant Genetics and Genomics: Crops and Models"*, Vol. 11: *Genetics and Genomics of the Saccharinae*. Springer, N.Y., pp. 367-389

Oguchi, T., Chikagawa, Y., Kodama, T., Suzuki, E., Kasahara, M., Akiyama, H., Teshima, R., Futo, S., Hino, A., Furui, S. and Kitta, K. (2009), "Investigation of residual DNAs in sugar from sugar beet (*Beta vulgaris* L.)", *J. Food Hyg. Soc. Japan*, Vol. 50, pp. 42 – 46

Potter, R. L., Bacheller J. D., et al. (1990), "Isolation of proteins from commercial beet sugar preparations", *J. Agr. Food Chem.* Vol. 38, pp. 1498-1502

Schell M.A., Zhao P., Wells L. (2011), "Outer membrane proteome of *Burkholderia pseudomallei* and *Burkholderia mallei* from diverse growth conditions", *J Proteome Res.* Vol. 10(5), pp. 2417-24

Taylor, G.O., Joyce, P.A., Sedl, J.M. and Smith, G.R. (1999), "Laboratory crystallised sugar from genetically engineered sugar cane does not contain transgene DNA", *Proc Aust. Soc. Sugar Cane Technol.*, Vol. 21, pp. 502

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Monitoring genetically modified soybean along the industrial soybean oil extraction and refining processes by polymerase chain reaction techniques

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ABSTRACT

In the present work, the extraction and detection of DNA along a complete industrial soybean oil processing chain was described to monitor the presence of Roundup Ready® (RR) soybean. The analysed samples comprised all the steps prior to industrial oil extraction, namely, raw, cracked, laminated and expanded seeds, and the defatted flour as a sub-product. The samples collected at the refining unit included the crude oil, degummed/neutralised, washed, bleached and deodorised oil, as final product. The amplification of soybean lectin gene by end-point polymerase chain reaction (PCR) was successfully achieved in all the steps of extraction and refining processes, until the fully refined soybean oil. The amplification of RR soybean by PCR assays using event-specific primers was also achieved for all the extraction and refining steps, except for the intermediate steps of refining (neutralisation, washing and bleaching) possibly due to sample instability. The real-time PCR assays using specific probes confirmed all the results and proved that it is possible to detect and quantify genetically modified organisms in the fully refined soybean oil. To our knowledge, this has never been reported before and represents an important accomplishment regarding the traceability of genetically modified organisms in refined oils.

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1. Introduction

The detection of genetically modified organisms (GMO) is one of the most important consumer concerns regarding food safety and quality. The recent progress in biotechnology and particularly in genetic engineering has revolutionised the agriculture by the introduction of new characteristics, such as the herbicide tolerance in soybean. The major GM crop species is soybean (*Glycine max*), accounting for almost 53% of the total world's GM planted area in 2008 (James, 2008). As an important source of protein and vegetable oil, presently the use of soybean GM seeds for soybean oil production has been continuously increasing and according to Soy Stat® data (Soy Stats, 2009) soybean represented 30% of total oil consumption in 2007.

The European Union (EU) regulations based on precautionary principles established both the legal basis for the approval procedure of GMO and the post-market traceability and labelling requirements for GMO and GMO-derived food and feeds (Regulations (EC) No. 1829/2003, 1830/2003). It requires any food containing more than 0.9% GM content to be labelled as such. The most accepted analytical methods for GMO detection are based on DNA techniques such as polymerase chain reaction (PCR), since the protein-based methods are not reliable for highly processed food analysis. However, the successful DNA amplification methods depend on the efficiency of DNA extraction protocols, which is considered a critical task in the analysis of complex and/or very processed food matrices. In the specific case of vegetable oils, a problem to overcome is the minute amounts of DNA in the sample to be extracted. Moreover, as most vegetable oils, crude soybean oil must be refined before consumption (Martin, Milinsk, Visentainer, Matsushita, & De-Souza, 2007).

The refining process, either physical or chemical, allows the elimination of unacceptable substances, such as phospholipids, free fatty acids (FFA) and pigments, which may led to an inferior quality of the final product. The chemical refining of crude soybean oil includes a first step of degumming, in which phosphoric acid is added to the oil to remove phospholipids and mucilaginous gums, followed by neutralisation with concentrated NaOH to eliminate FFA and a washing step to remove the formed soaps. Subsequently, the oil is submitted to a bleaching step using activated carbon or clays and to a final deodorisation step using steam at reduced pressure to eliminate the remaining FFA and promoting the thermal destruction of peroxides. All these steps, especially the heat treatments, the use of activated clays and pH variations, may affect the quantity and quality of the DNA that remains in the fully refined oil (Gryson, Ronsse, Messens, De Loose, & Verleyen et al., 2002). Thus, when dealing with surveillance testing of GMO in highly processed

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foods, such as the case of vegetable oils, researchers must face with major analytical challenges.

Several works have demonstrated the applicability of DNA markers as tools for authenticity assessment of olive oils (Breton, Claux, Metton, Skorski, & Bervillé, 2004; Consolandi et al., 2008; Doveri, Sullivan, & Lee, 2006; Martins-Lopes, Gomes, Santos, & Guedes-Pinto, 2008; Muzzalupo, Pellegrino, & Perri, 2007; Muzzalupo & Perri, 2002). However, virgin olive oil is exclusively obtained by mechanical means without any further treatment, not posing some difficulties found in refined oils. Regarding other vegetable matrices, previous reports evidenced positive results in the extraction and amplification of DNA from crude vegetable oils, such as rapeseed (Hellebrand, Nagy, & Mörsel, 1998) and soybean (Gryson, Messens, & Dewettinck, 2004; Gryson et al., 2002; Pauli, Liniger, & Zimmermann, 1998). Concerning the positive detection of DNA in fully refined vegetable oils, very few studies are available. Doveri and Lee (2007), when applying different protocols for DNA extraction on a range of processed foods were able to amplify DNA from commercial sunflower and maize oils. Bogani et al. (2009) achieved the qualitative detection of RR soybean at an industrial soybean processing chain until degummed oil and lecithin, but no data for the subsequent steps and fully refined soybean oil. Recently, we have optimised and compared different DNA extraction protocols, which enabled to obtain amplifiable soybean DNA from fully refined vegetable oils (Costa, Mafra, Amaral, & Oliveira, submitted for publication). Considering the previous successful results, the aim of the present work was to isolate DNA from a complete industrial soybean oil processing chain to monitor, qualitative and quantitatively, the presence of Roundup Ready® soybean.

2. Materials and methods

2.1. Samples

The extraction and chemical refining processes were carried out at industrial scale (Sovena, Portugal). Representative samples were collected along the soybean oil production line (Fig. 1). The first industrial step consisted on cleaning and removing impurities from the soybean seeds. At this point the first sample (whole seed) was collected. The following steps consisted on cracking, cleaving and expelling-extruding the soybean seeds. For each one, a sample was collected, corresponding to cracked, laminated and extruded soybean seeds, respectively. The extruded material was then submitted to solvent extraction with *n*-hexane obtaining the crude oil, which was conducted to the refining unit, and the defatted flour, as sub-product for animal feed. The crude oil was acidified with phosphoric acid (1.1 L per oil ton) followed by neutralisation with NaOH (28.5–29.5 mol/L) and centrifugation for the removal of phospholipids and FFA (neutralised oil sample). Subsequently, the oil was washed with water (80 °C) and centrifuged to remove the remaining soaps (washed oil sample) and bleached (with activated clay and activated charcoal, 100 °C, 600 mm Hg) to clean the oil from impurities like pigments (bleached oil sample). The final step was the deodorisation phase (240 °C at reduced pressure, during approximately 2 h) in which unpleasant odours were removed (deodorised oil sample). The collected oil samples were kept in dark bottles filled to the top, nitrogen flushed and then stored in the dark until analysis.

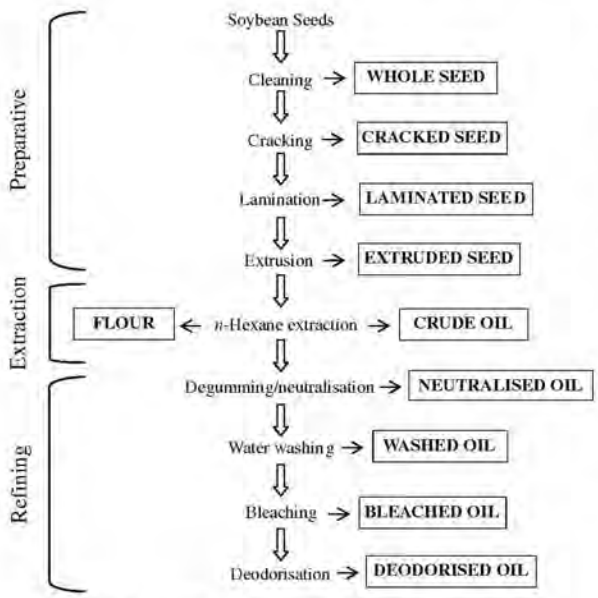

Fig. 1. Sampling from the soybean oil production chain.

Certified reference material (CRM) from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) were used as standard materials for testing the presence of GMO soybean, and consisted of dried soybean powder with 0.1%, 1% and 5% (w/w) of Roundup Ready® soybean (Fluka, Buchs, Switzerland).

2.2. DNA extraction

The DNA extraction protocols were based on previously developed methods, namely, Wizard, for seed materials (whole seeds, cracked, laminated, extruded seeds and defatted flour), and nucleospin for oil samples (crude, degummed/neutralised, washed, bleached and deodorised oils) (Fig. 1).

2.2.1. Wizard extraction method

The Wizard method consisted on the use of the Wizard® columns and resin (Promega, Madison, WI, USA) as described by Lipp, Brodmann, Pietsch, Pauwels, and Anklam (1999), with some modifications introduced by Mafra, Silva, Moreira, Silva, and Oliveira (2008). Seed materials were previously triturated and homogenised prior to extraction.

To the flour samples (100 mg), it was added 860 μL of TNE extraction buffer (10 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 1% SDS), 100 μL of 5 mol/L guanidine hydrochloride solution and 40 μL of proteinase K solution (20 mg/mL). The mixtures were incubated at 60 °C for 3 h, with occasional stirring. After incubation, the mixtures were centrifuged for 15 min (18,514g, 4 °C) and 500–600 μL of supernatant were collected and mixed with 1 mL of Wizard® DNA purification resin (Promega, Madison, WI, USA). Each mixture was then pushed along a column mounted with a 2 mL syringe. The DNA–resin mix was washed once with 2 mL isopropanol solution (80%, v/v), followed by a centrifugation for 2 min at 10,000g. The column was then dried for 5 min at room temperature and mounted on a new sterile reaction tube. After incubation for 1 min with 50 μL of 0.1× Tris–EDTA buffer (10 mmol/L Tris, 1 mmol/L EDTA) at 70 °C, the column was eluted by centrifugation (1 min, 10,000g). All the extracts were kept at −20 °C until further analysis. The extractions were done in duplicate assays for each sample.

2.2.2. Nucleospin method

Prior to DNA extraction, all the oil samples were pre-concentrated by centrifugation from a total of 200g of oil carefully weighted to four sterile centrifuge tubes. The oil samples were then centrifuged at 18,514 g, for 30 min at 4 °C. The supernatant was discarded and the residual pellet was transferred to a set of

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four sterile 2 mL reaction tubes. The reaction tubes were centrifuged for 10 min (18,514 g, 4 °C), the supernatant was discarded and the pellet was then submitted to DNA extraction.

The Nucleospin® food kit (Macherey–Nagel, Düren, Germany) was performed according to the manufacturer instructions with some minor modifications. To the pre-concentrated oil samples (pellet collected) in 2 mL × 4 reaction tubes, it was added to each tube 550 μL of lysis solution CF pre-heated at 65 °C and 10 μL of proteinase K (20 mg/mL). The mixture was incubated at 65 °C for 1 h with occasional stirring and centrifuged for 10 min (18,514 g, 4 °C). The supernatants were transferred (400–500 μL) to new sterile reaction tubes and the same volume of precipitation solution C4 and 0.6 volume parts of ethanol 100% were added. The mixtures were homogenised by inversion and the volumes of the 4 tubes were eluted through one spin column by centrifugation (1 min, 13,000 g). The column was washed with 2 × 400 μL of solution CQW, 2 × 700 μL and 2 × 200 μL of C5 solution followed by 1 min centrifugation (10,000 g) after the first washings and a 2 min centrifugation after the final one. The DNA was eluted from the column by the addition of 50 μL of CE solution at 70 °C, followed by 5 min incubation and centrifugation (2 min, 10,000 g). All the extracts were kept at −20 °C until further analysis. The extractions were done in duplicate assays for each sample.

2.3. DNA purity and quality

The quality and purity of extracted DNA was analysed by spectrophotometry using a Nanophotometer™ IMPLEN (GmbH, Munich, Germany). DNA concentration was determined by UV absorbance at 260 nm. The purity of the extracted DNA was determined by the ratio of the absorbance at 260 nm and 280 nm.

2.4. Oligonucleotide primers and probes

The oligonucleotide primers and probes used in this work are presented in Table 1. The primers and probes were synthesised by Eurofins MWG Operon (Ebersberg, Germany).

For the event specific detection of RR soybean, new primers were specifically designed targeting the plant genome and the NOS terminator junction zone (RRS-3J1/RRS-3J3). The primers were designed based on software accessible in http://seq.yeastgenome.org and the nucleotide sequences were submitted to a BLASTn sequence similarity search, which confirmed primer specificity.

2.5. Qualitative PCR

The amplifications by polymerase chain reaction (PCR) were carried out in 25 μL total reaction volume containing 2 μL or 4 μL of DNA extracts of seed materials or oil samples, respectively, 15 mmol/L Tris–HCl (pH 8.3), 50 mmol/L KCl, 0.5 mmol/L of each primer for lectin gene (LE1/LE2) or 0.4 mmol/L of each primer for RR soybean (RRS-3J1/RRS-3J3), 0.2 mmol/L of each dNTP (Invitrogene, Carlsbad, CA, USA), 2 mmol/L $MgCl_2$ and 1 U of DNA polymerase AmpliTaq Gold® (Applied Biosystems, Branchburg, NJ, USA).

The PCR amplifications were performed in a thermal cycler PTC-100 (MJ Research, Inc., Watertown, MA, USA) using the following program: an initial denaturation at 94 °C for 4 min, with 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 4 min.

The amplified fragments were analysed by electrophoresis in a 2.0% agarose gel carried out in TAE buffer (40 mM Tris–acetate, 1 mM EDTA) for 60 min at 120 V, stained with ethidium bromide (0.4 μg/mL for 5 min) and destained in distilled water for 30 min. The agarose gel was visualised under UV light and a digital image was obtained using a Kodak Digital Science™ DC120 (Rochester, NY, USA). Each extract was amplified at least in duplicate assays.

2.6. Real-time PCR

The amplifications by real-time PCR were carried out in 20 μL containing 2 μL or 4 μL of DNA extracts of seed materials or oil samples, respectively, 1× of iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA, USA), 900 nmol/L of each primer and 100 nmol/L of each probe (Table 1). The real-time PCR assays were performed on a fluorometric thermal cycler iCycler iQ™ Real-time Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 95 °C for 1 min; 50 cycles at 95 °C for 30 s and 60 °C for 1 min, with collection of fluorescence signal at the end of each cycle. Data was collected and processed using an iCycler iQ™ Real-Time Detection System Software version 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was amplified in triplicate and each dilution standard in duplicate assays.

Table 1
Primers and probes used in qualitative and real-time PCR.

Name	Sequence (5′–3′)	Target	Fragment length (bp)	References
GM03 GM04	GCC CTC TAC TCC ACC CCC ATC C GCC CAT CTGCAA GCC TTT TTG TG	Lectin gene	118	Querci, Maretti, and Mazzara (2006)
LE1 LE2	AGC AAT GGC TAC TTC AAA G TGA GTT TGC CTT GCT GGT CAG T	Lectin gene	103	Mafra et al. (2008)
GM07 GM08	ATC CCA CTA TCC TTC GCA AGA TGG GGT TTA TGG AAA TTG GAA	CP4 EPSPS	169	Querci et al. (2006)
RRS-3J1[a] RRS-3J3[a]	TCT ACA TAT AGC TTC TCG TTG AAC TTC TCG ACG ATG GCC G	NOS 3′UTR/ plant	106	This work
Lectin-F Lectin-R Lectin-TMP probe	TCC ACC CCC ATC CAC ATT T GGC ATA GAA GGT GAA GTT GAA GGA FAM-AAC CGG TAG CGT TGC CAG CTT CG-BHQ2[b]	Lectin gene	81	ISO 21570 (2005)
RRS-F RRS-R RRS-TMP probe	GCC ATG TTG TTA ATT TGT GCC AT GAA GTT CAT TTC ATT TGG AGA GGA C FAM-CTT GAA AGA TCT GCT AGA GTC AGC TTG TCA GCG-BHQ2[b]	CTP/35S junction	83	ISO 21570 (2005)

[a] Genbank AJ308515.
[b] FAM – 6-carboxyfluorescein; BHQ2 – black hole quencher 2.

3. Results and discussion

3.1. Qualitative and quantitative analysis of extracted DNA

Two different DNA extraction protocols were used in this work, depending on the analysed matrix. For seed materials several protocols could be successfully applied, thus the Wizard method was chosen for its simplicity and economy. Concerning the DNA extraction of oil matrices, the protocol was critically chosen and optimised. Since previous work demonstrated that the Nucleospin method with a pre-concentration step could be used to obtain amplifiable DNA from refined oils (Costa et al., submitted for publication), this was the protocol chosen for all the steps of oil refining.

DNA concentration and purity were estimated by UV spectrophotometry measuring the absorbance at 260 nm (A_{260}) and the A_{260}/A_{280} absorbance ratio, respectively, using a nanophotometer. Previous results showed that by spectrofluorimetry using the Quant-it™-Picogreen® dsDNA Kit it was not possible to detect DNA from oil extracts. Table 2 presents the results of DNA concentration and purity of the extracts obtained from the samples collected along the industrial soybean oil production chain. The DNA extracts of the seed materials prior and after the soybean oil extraction presented, generally, high DNA yields (71.6–95.7 μg) and purity ($A_{260}/A_{280} > 1.6$). Regarding oil samples, crude oil produced the best extracts due to the relatively high yield (1415 ng) comparing with the other oil extracts and the high purity ($A_{260}/A_{280} = 1.95$). The bleached oil produced also a relatively high DNA yield (1625 ng), but with low purity. The other oil extracts showed similar and much lower yields (average 225 ng), which was expected from previous results from the extraction of fully refined oils (Costa et al., submitted for publication).

3.2. Qualitative PCR amplification

On a first stage, the amplifiability of extracted DNA was evaluated by PCR targeting the soybean lectin gene. Using the primers GM03/GM04 (Table 1), all the extracts derived from soybean seed materials collected in the pre-extraction steps produced strong PCR bands with the expected fragment size of 118 bp (data not shown). When amplifying oil extracts, an expected fragment was observed only the in crude oil extract (Fig. 2, Lane 1), since after degumming/neutralisation (Lane 2) no PCR products could be detected. To overcome this result, the primers LE1/LE2 (Table 1) were used to amplify a slightly shorter PCR fragment (103 bp). Fig. 3A shows the amplification results of PCR targeting the lectin gene using LE1/LE2 primers, evidencing the strong positive detection of soybean in all steps of refining process. It should be noted that this was performed using samples of 200 g of oil in all the steps of refining, though prior results proved that only about 30 g of crude oil were enough to obtain amplifiable DNA. This result

Table 2
Concentration and purity of DNA extracts of samples from all the steps of soybean oil extraction and refining processes.

Samples	DNA concentration (ng/μL)	Purity A_{260}/A_{280}[a]
Seed	883.5 ± 16.7	1.87 ± 0.01
Cracked seed	956.5 ± 12.0	1.78 ± 0.04
Laminated seed	847.5 ± 3.50	1.83 ± 0.01
Extruded seed	716.5 ± 4.90	1.73 ± 0.00
Flour	760.0 ± 2.80	1.68 ± 0.02
Crude oil	28.3 ± 0.4	1.95 ± 0.02
Neutralised oil	4.50 ± 0.00	1.61 ± 0.08
Washed oil	3.90 ± 1.27	1.21 ± 0.01
Bleached oil	32.5 ± 7.1	1.23 ± 0.09
Deodorised oil	5.13 ± 1.24	1.27 ± 0.08

[a] A_{260} – absorbance at 260 nm; A_{280} – absorbance at 280 nm.

Fig. 2. PCR amplification targeting the soybean lectin gene of crude and degummed/neutralised oils using primers GM03/GM04. Lane 1 – crude oil; lane 2 – neutralised oil; B – blank extraction; N – negative control; M – 100 bp ladder (Bioron, Ludwigshafen, Germany).

agrees with the previous successful amplification of DNA from fully refined oils (Costa et al., submitted for publication) using the same set of primers. Experiments using blank extractions as controls confirmed the absence of DNA contamination (Fig. 3A, Lane B). In contrast to the difficulties in obtaining amplifiable DNA after the first steps of soybean oil refining reported by other authors (Gryson et al., 2002; Gryson et al., 2004; Pauli et al., 1998), namely, after the degumming step where it is considered that DNA is transferred to the water fraction (Gryson et al., 2002), the present results are of major relevance since DNA fragments were detected for the first time in all the steps of soybean oil refining process. Although Bogani et al. (2009) recently reported the detection of GMO along the complete industrial soybean oil production, they only attempted to detect DNA until the degumming step. After the subsequent steps where aggressive conditions for DNA take place, namely, increase of pH by neutralising with concentrated soda, centrifugations in the neutralisation and washing steps and high temperatures during washing (80 °C), bleaching (100 °C) and deodorisation (240 °C and reduced pressure), we were still able to detect amplifiable DNA.

To evaluate the presence of RR soybean along the oil refining, event-specific PCR primers (RRS-3J1/RRS-3J3) targeting also a short DNA fragment (106 bp) were used. In Fig. 3B are presented the PCR products obtained for RR soybean detection along the oil refining process. The results evidence the clear amplification of RR soybean in crude oil (Lane 1) and final deodorised oil (Lane 5). However, no amplification was obtained in the intermediate steps of degumming/neutralisation, washing and bleaching (Lanes 2–4). This was not expected, since all the extracts amplified positively for the lectin gene and they were all from oil samples collected in the same period and line of production. The low level of DNA concentration associated to the low purity of extracts after washing (Table 2) step might have contributed for this result. In our opinion, the instability of oil samples from the intermediate steps of processing might have contributed to a slightly higher level of DNA degradation than in the final stable deodorised product. Anyhow, the positive amplification of RR soybean in fully refined oil proves that it is possible to detect GMO in this type of processed samples.

3.3. Real-time PCR amplification

For confirmation of the qualitative PCR results as well as for quantitative analysis, all extracts were amplified by real-time

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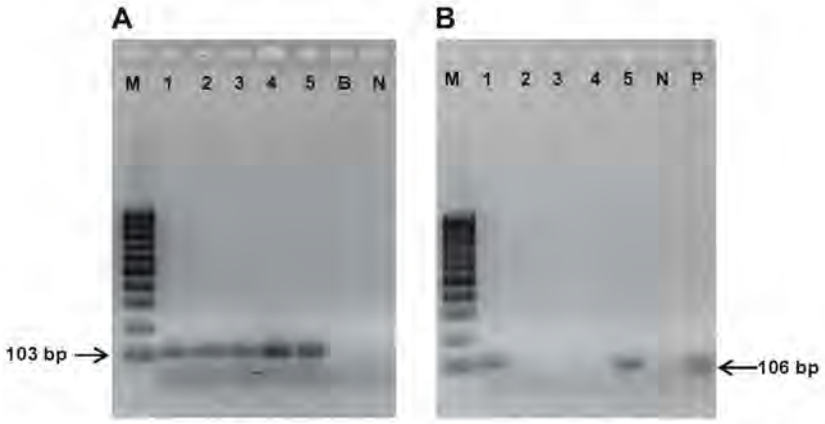

Fig. 3. PCR amplification of oil samples from refining steps. A – Soybean lectin gene detection using primers LE1/LE2. B – RR soybean detection using primers RRS-3J1/RRS-3J3. Lanes 1–5 – crude, neutralised, washed, bleached and deodorised oils, respectively; B – blank extraction; P – positive control (certified reference material of 0.1% RR soybean); N – negative control; M – 100 bp ladder (Bioron, Ludwigshafen, Germany).

Table 3
Real-time PCR results of samples from all the steps of soybean oil extraction and refining processes.

Samples	Lectin		RR		GMO (%)
	Ct[a]	DNA copies[b]	Ct[a]	DNA copies[b]	
Seed	27.73 ± 0.07	86327.4	29.17 ± 0.11	48348.1	56.0 ± 3.9
Cracked seed	27.54 ± 0.01	98672.6	29.29 ± 0.19	44867.7	45.5 ± 5.8
Laminated seed	29.14 ± 0.06	31946.9	30.70 ± 0.05	17551.6	54.9 ± 1.9
Extruded seed	27.06 ± 0.07	138495.6	28.97 ± 0.13	55132.7	39.8 ± 3.3
Flour	27.28 ± 0.06	119026.5	28.74 ± 0.02	64247.8	54.0 ± 0.6
Validation CRM[c] 1%	27.84 ± 0.00	79911.5	34.32 ± 0.19	823.0	1.03 ± 0.07
Crude oil	25.76 ± 0.32	7967.6	26.78 ± 0.59	4814.2	60.4 ± 13.3
Neutralised oil	29.96 ± 0.47	454.0	37.54 ± 0.66	4.2	ND[d]
Washed oil	31.74 ± 0.26	131.4	NA[e]	–	–
Bleached oil	29.86 ± 0.04	471.7	NA	–	–
Deodorised oil	27.56 ± 0.05	1778.8	29.59 ± 0.73	786.2	44.2 ± 15.0
Validation CRM[c] 1%	20.85 ± 0.09	229331.9	28.30 ± 0.07	2256.6	0.98 ± 0.05

[a] Ct – cycle threshold.
[b] Values are the average of three replicate assays.
[c] CRM – certified reference material (IRMM).
[d] ND – not determined.
[e] NA – no detectable amplification.

PCR using specific fluorescent probes proposed by ISO 21570 (2005). The lectin gene used as a reference gene was amplified with oligonucleotide primers Lectin-F/Lectin-R and probe Lectin-TMP, and the construct-specific RR soybean was amplified with the primers RRS-F/RRS-R and probe RRS-TMP (Table 1). For quantitative analysis, standard curves were prepared using a certified reference material from IRMM (5% RR soybean) serially diluted (1/3). The standard curves used for the higher concentration DNA extracts (seed materials) ranged from 133 ng to 0.1826 ng for lectin gene and from 20 ng to 0.247 ng for RR soybean with linear correlation coefficients (R^2) of 0.997 and 0.995, and PCR efficiencies of 102.8% and 93.4%, respectively. The standard curves used for the lower concentration DNA extracts (oils) ranged from 133 ng to 0.1826 ng for lectin gene and from 20 ng to 0.02744 ng for RR soybean with linear correlation coefficients (R^2) of 0.994 and 0.996, and PCR efficiencies of 105.0% and 96.0%, respectively.

Real-time PCR amplification results for all samples collected along the soybean oil extraction and refining steps are presented in Table 3. For samples collected prior to oil extraction, including the flour as sub-product, the results confirmed the use of GM seeds with an average proportion of 50.7%. The oil extracts amplified positively for lectin gene in all the steps of refining, which confirmed all the positive results of the end-point PCR assay (Fig. 3A). The detection of RR soybean was also in good agreement with the end-point PCR results, since the positive results for crude and final deodorised oils were confirmed (Fig. 3B). Besides, the GMO percentages of crude and deodorised oils were according with the values of the pre-extraction seed materials (Table 3). The negative amplifications for washed and bleached oils or very low detection level for neutralised oil are in good agreement with the end-point PCR results. As verified by the very low number of DNA copies for lectin gene in those extracts, it would be expectable to have difficulties in amplifying RR soybean.

4. Concluding remarks

In this study we have demonstrated for the first time the detection of amplifiable DNA in all the stages of chemical refining of crude soybean oil by end-point and real-time PCR techniques. Moreover, we have also proved that it is possible to detect RR soybean along the refining process and that it is possible to quantify the amplifiable DNA in crude and final deodorised soybean oils. This was successfully accomplished by using start samples of

200 g in all steps of refining and by using suitable primers to amplify short PCR fragments. These findings are of major importance for the traceability of GMO at industrial scale.

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References

Bogani, P., Minunni, M., Spiriti, M. M., Zavagliam, M., Tombellini, S., Buiatti, M., et al. (2009). Transgenes monitoring in an industrial soybean processing chain by DNA-based conventional approaches and biosensors. *Food Chemistry, 113*, 658–664.

Breton, C., Claux, D., Metton, I., Skorski, G., & Bervillé, A. (2004). Comparative study of methods for DNA preparation from olive oils samples to identify cultivar SSR alleles in commercial oil samples: Possible forensic application. *Journal of Agricultural and Food Chemistry, 52*, 531–537.

Consolandi, C., Palmieri, L., Severgnini, M., Maestri, E., Marmiroli, N., Agrimonti, C., et al. (2008). A procedure for olive oil traceability and authenticity: DNA extraction, multiplex PCR and LDR-universal array analysis. *European Food Research Technology, 227*, 1429–1438.

Costa, J., Mafra, I., Amaral, J. S., & Oliveira, M. B. P. P. (submitted for publication). Detection of genetically modified soybean DNA in refined vegetable oils.

Doveri, S., & Lee, D. (2007). Development of sensitive crop-specific polymerase chain reaction assays using 5S DNA: Applications in food traceability. *Journal of Agricultural and Food Chemistry, 55*, 4640–4644.

Doveri, S., Sullivan, D. M. O., & Lee, D. (2006). Non-concordance between genetic profiles of olive oil and fruit: A cautionary note to use of DNA markers for provenance testing. *Journal of Agricultural and Food Chemistry, 54*, 9221–9226.

European Commission (2003). Regulation (EC) No. 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed. *Official Journal of the European Union, L 268*, 1–23.

European Commission (2003). Regulation (EC) No. 1830/2003 of the European Parliament and of the Council of 22 September 2003 concerning the traceability of food and feed products produced from genetically modified organisms and amending directive 2001/18/EC. *Official Journal of the European Union, L 268*, 24–28.

Gryson, N., Messens, K., & Dewettinck, K. (2004). Influence of different oil-refining parameters and sampling size on the detection of genetically modified DNA in soybean oil. *Journal of the American Oil Chemists' Society, 81*, 231–234.

Gryson, N., Ronsse, F., Messens, K., De Loose, M., Verleyen, T., & Dewettinck, K. (2002). Detection of DNA during the refining of soybean oil. *Journal of the American Oil Chemists' Society, 79*, 171–174.

Hellebrand, M., Nagy, M., & Mörsel, J. T. (1998). Determination of DNA traces in rapeseed oil. *Zeitschrift für Lebensmittel – Untersuchung und Forschung A, 206*, 237–242.

International Standard ISO 21570 (2005). *Foodstuffs – Methods of analysis for the detection of genetically modified organisms and derived products – Quantitative nucleic acid based methods*. Geneva: International Organization for Standardization.

James, C. (2008). *Global Status of Commercialized Biotech/GM Crops: 2008. ISAAA Brief No. 39*. New York, Ithaca: ISAAA.

Lipp, M., Brodmann, P., Pietsch, K., Pauwels, J., & Anklam, E. (1999). IUPAC collaborative trial study to detect genetically modified soy beans and maize in dried powder. *Journal of the AOAC International, 82*, 923–928.

Mafra, I., Silva, S. A., Moreira, E. J. M. O., Silva, C. S. F., & Oliveira, M. B. P. P. (2008). Comparative study of DNA extraction methods for soybean derived food products. *Food Control, 19*, 1183–1190.

Martin, C. A., Milinsk, M. C., Visentainer, J. V., Matsushita, M., & De-Souza, N. E. (2007). Trans fatty acid process forming: A review. *Anais da Academia Brasileira de Ciências, 79*, 343–350.

Martins-Lopes, P., Gomes, S., Santos, E., & Guedes-Pinto, H. (2008). DNA markers for Portuguese olive oil fingerprinting. *Journal of Agricultural and Food Chemistry, 56*, 11786–11791.

Muzzalupo, I., Pellegrino, M., & Perri, E. (2007). Detection of DNA in virgin olive oils extracted from destoned fruits. *European Food Research Technology, 224*, 469–475.

Muzzalupo, I., & Perri, E. (2002). Recovery and characterisation of DNA from virgin olive oil. *European Food Research Technology, 214*, 528–531.

Pauli, U., Liniger, M., & Zimmermann, A. (1998). Detection of DNA in soybean oil. *Zeitschrift für Lebensmittel – Untersuchung und Forschung A, 207*, 264–267.

Querci, M., Maretti, M., & Mazzara, M. (2006). Qualitative detection of MON810 maiza, Bt-176 maize and Roundup Ready® soybean by PCR. In M. Querci, M. Jermini, G. V. Eede (Eds.), *Training course on the analysis of food samples for the presence of genetically modified organisms*, (Session 9). European Commission DG-JRC. Luxembourg: Office for Official Publications of the European Communities <http://gmotraining.jrc.it>.

Soy Stats (2009). <http://www.soystats.com/2008/page_35.htm> Accessed 14.01.09.

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Report

Investigation of Residual DNAs in Sugar from Sugar Beet (*Beta vulgaris* L.)

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Genetically modified (GM) sugar beets have been bred for use as food and animal feed. To evaluate the applicability of GMO analyses to beet sugar products, we investigated residual DNA in eight sorts of in-process beet sugar samples and commercial beet sugar products. Polymerase chain reaction (PCR) analyses with taxon-specific primers indicated that sugar beet DNA was degraded at an early stage of sugar processing, and no PCR amplification was detected from the investigated sugar products because of low DNA recovery and/or PCR inhibition.

Key words: genetically modified (GM); sugar beet (*Beta vulgaris* L.); deoxyribonucleic acid (DNA); polymerase chain reaction (PCR); taxon specific DNA

Introduction

Recombinant DNA technologies have been increasingly used in modern farming and are thought to offer various advantages. The global area of genetically modified (GM) crops exceeded 120 million hectares in 2007, and is expected to continue to rise[1]. GM crops have been authorized for use as food and/or animal feed in many countries based on their own criteria for safety assessment. However, consumers have demanded appropriate information and labeling for foods derived from GM crops. Thus, labeling systems have been introduced for GM foods in the European Union (EU), Korea, Japan, Australia and other countries, and these systems are distinct from each other[2]. In addition, many countries have been seeking ways for the coexistence of cultivation of conventional crops and GM crops. In these situations, scientifically sound GMO detection methods have become more important. Sugar beet is a major agricultural crop, used as the raw material for refined sugar, especially in cool regions. GM sugar beets have also been bred and authorized for food and/or feed applications by many countries. Therefore it is desirable to survey the commercial distribution of GM sugar beets and/or their processed foods. However, it has not been established whether or not sufficient amounts and/or quality of DNAs for DNA extraction-based analyses remain in refined beet sugar products. In this study, we investigated residual DNA in commercial beet sugar products and assessed the appropriateness of GMO analysis methods for processed sugar beet products for regulatory purposes.

Materials and Methods

Materials

Fresh sugar beet and eight sorts of in-process samples of sugar beets were kindly provided by the Japan Beet Sugar Association. The sampling points of the in-process samples are indicated in Fig. 1. Eight sorts of commercial beet sugar samples were purchased from markets in Tokyo and Sapporo in Japan. Nineteen varieties of sugar beets and four plants closely related to sugar beets were provided by the National Agricultural Research Center for the Hokkaido Region, and used for the specificity tests of PCR primers.

DNA extraction

For the PCR experimental controls, DNAs were extracted from leaves or aerial parts of seedlings with a DNeasy® plant Maxi kit (QIAGEN, Hilden, Germany) according to the attached protocol. DNA extraction from in-process samples and beet sugar products was performed using an anion exchange column, Genomic-tip 20/G (QIAGEN), and the experimental pro-

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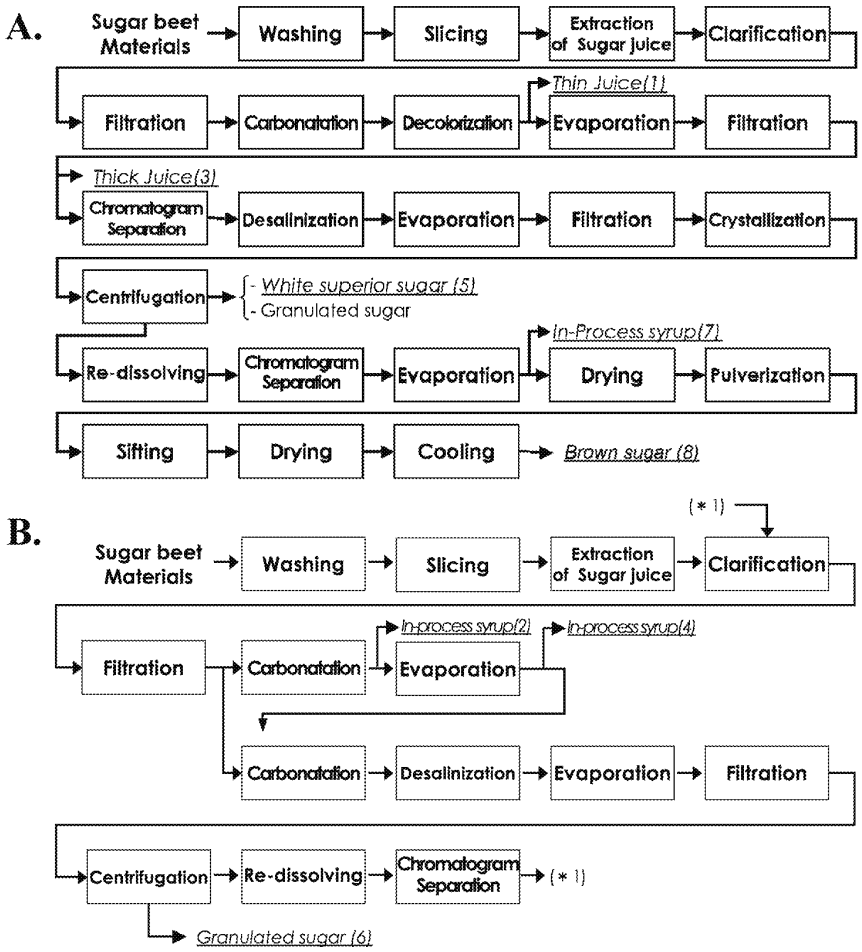

Fig. 1. Flow charts of beet sugar processes

Sugar beet materials are washed and sliced into thin strips called cassettes, which go through a diffuser machine to extract raw sugar juices. The raw juices are clarified and filtered to remove beet pulp. Filtered raw juices are mixed with "milk of lime", which consists of calcium oxide and carbon dioxide gas, and non-sugar components in the juices are precipitated with the calcium carbonate. Then, the supernatant is called thin juice. The thin juice is purified and evaporated to obtain thicker juice. The thick juice goes into a crystallizer tank and fine sugar crystals grow. The crystallized sugar is purified to fine sugar products, such as white superior sugar and granulated sugar. Much sugar remains in the syrup, and then chromatographic separation is performed on the syrup to produce brown sugar. The processes shown here are two examples (flow chart A and B) and there are some differences in processing from company to company. The underlined terms indicated the points at which specimens of the in-process products were taken for use in this study, and the numbers in parentheses correspond to the numbers in Table 1 and Fig. 4.

cedure generally followed the Japanese standard method for GMO analysis on food items with some modifications[3],[*1]. Extracted DNAs were finally resuspended in 40 μL of sterile distilled water. All extraction experiments were performed in a clean laboratory with designated operators, and two independent extracts were made from the in-process samples and beet sugar products.

Estimation of amount and quality of extracted DNAs

The amount and quality of the extracted DNA solutions was estimated from the ultraviolet (UV) absorption spectrum measured by a UV spectrophotometer, ND-1000 (NanoDrop® Technologies, Wilmington, DE, USA). 1.5 μL of each undiluted DNA extract was directly subjected to UV measurement, and the UV absorptions at 230 nm, 260 nm, and 280 nm were observed. In addition, as the experimental control, we also performed DNA quantitation using the Qant-iT™ PicoGreen® dsDNA quantitation reagent (Molecular Probes, Eugene, OR, USA) and Cytofluore® 2350 (Millipore, Billerica, MA, USA). The experimental proce-

[*1] Notification No. 110 (Mar. 27, 2001), Department of Food Safety, MHLW, Japan (2001).

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Table 1. Quality of the extracted DNA from in-process samples and beet sugar produsts

#	Type	Sample[a]	260 nm/280 nm[b]	260 nm/230 nm[c]	Conc. (ng/μL)[d]	Conc. (ng/μL)[e]
1	In-process samples	Thin juice	2.0	0.28	13.4	8.36
2		In-process syrup	1.2	0.08	3.3	0.72
3		Thick juice	2.3	0.07	3.0	0.58
4		In-process syrup	1.7	0.09	3.8	0.83
5		White superior sugar	1.2	0.08	2.2	0.36
6		Granulated sugar	1.7	0.09	3.0	−0.16
7		In-process syrup	1.7	0.07	3.1	−0.03
8		Brown Sugar	1.3	0.10	4.3	0.01
9	Commercial beet	Brown sugar (1)	2.8	0.09	4.3	0.39
10	sugar products	Brown sugar (2)	4.0	0.08	3.7	0.85
11		Syrup-type product added oligosaccharide	2.3	0.06	2.3	0.70
12		Granulated sugar (1)	2.7	0.06	2.9	0.72
13		White superior sugar	20.3	0.07	2.7	0.73
14		Brown sugar (3)	2.2	0.06	2.3	0.73
15		Bleached brown sugar	1.2	0.07	3.5	0.68
16		Granulated sugar (2)	5.0	0.06	2.6	0.66

[a] DNA extractions were performed with two independent replications, and UV absorptions are given as the means of them.
[b] The ratios of DNA solution in good condition usually range from 1.7 to 2.0.
[c] The ratios of DNA solution in good condition are usually more than 0.6.
[d] Calculated concentrations estimated from UV absorptions.
[e] Calculated concentrations estimated using the Qant-iT™ PicoGreen® reagent.

dures followed the manufacturers' protocols.

Qualitative PCR analysis

The GeneAmp® PCR system 9700 (Applied Biosystems; ABI, Foster City, CA, USA) was used in the max mode, and the PCR mixture, in a final volume of 25 μL, consisted of 1X PCR buffer II (ABI), 0.2 mM dNTPs (ABI), 1.5 mM $MgCl_2$ (ABI), 0.025 U AmpliTaq® Gold DNA polymerase (ABI), 0.5 μM each primer and DNA sample. Twenty-five ng (2.5 μL of 10 ng/μL) of template DNA, as calculated from the UV absorption at 260 nm, was used for PCR analysis unless otherwise described. When the concentration of extracted DNA was not enough, the maximum volume (17,875 μL) of undiluted DNA extract was used for the reaction. The primer pairs used in this study were as follows: primer pair 1; 5′-GCCCCCAAAAACCCTTCA-3′ and 5′-GGGCAATTTGGTAGGCTTCTT-3′, and primer pair 2; 5′-ATCCCTGCAGCCATCAGTGA-3′ and 5′-ACCAGTAAGCCACTCAACAGTCAA-3′. As an inhibition assay of PCR, we observed amplification from each reaction mixture spiked with 260 pg of extracted DNA from the sugar beet plant (cv. Skane). Twenty-five μL of PCR mixture consisted of 1X PCR buffer II (ABI), 0.2 mM dNTPs (ABI), 1.5 mM $MgCl_2$ (ABI), 0.025 U AmpliTaq® Gold DNA polymerase (ABI), 0.5 μM each primer, 16,875 μL of DNA extraction, and 1 μL of 260 pg/μL spike DNA.

The qualitative PCR reactions was performed on a thermal cycler, the Silver 96-Well GeneAmp® PCR System 9700 (ABI) in Max mode, according to the following step-cycle program: pre-incubation at 95℃ for 10 min; 40 cycles consisting of denaturation at 95℃ for 0.5 min, annealing at 60℃ for 0.5 min and extension at 72℃ for 0.5 min; followed by a final extension at 72℃ for 7 min. After the amplification, PCR products were electrophoresed on 3% agarose gels buffered with Tris-Acetate-EDTA (TAE) solution.

Results and Discussion

Yield and quality of extracted DNA from in-process samples and beet sugar products

DNA was extracted from 1 g of each in-process or beet sugar product sample with the anion exchange column, Genomic-tip 20/G (QIAGEN), which we have been using for the DNA extraction of highly processed foods[3]. The yield and quality of the extracted DNA solution was estimated from the UV absorption spectrum. The UV absorption ratios at 260 nm/280 nm of most samples were out of the optimal range of 1.7 to 2.0, which indicated poor quality of DNA (Table 1). Moreover, absorption ratios of 260 nm/230 nm ranged from 0.06 to 0.28, which implied that sugars contaminated the extracted DNA solutions (Table 1). Based on the UV absorption at 260 nm, the calculated concentrations of extracted DNAs ranged from 2.2 ng/μL (white superior sugar) to 13.4 ng/μL (thin juice) and from 2.3 ng/μL (syrup-type product with added oligosaccharide, and brown sugar #3) to 4.3 ng/μL (brown sugar #1) for in-process samples and commercial products, respectively (Table 1). Following the Japanese standard method for GM analyses, we generally used 25 ng of DNA, *i.e.*, 2.5 μL of 10 ng/μL diluted DNA, for quantitative PCR analysis. Thus, these yields were very low. In addition, the UV absorption measurements indicated that contamination with nucleic acids and/or other substances unrelated to PCR amplification was probably presnt. In fact, fluorometric quantitation of double-

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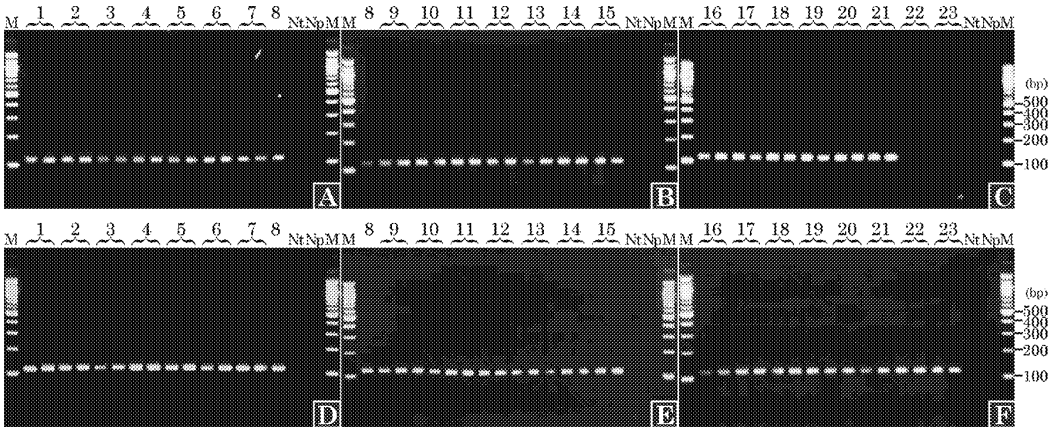

Fig. 2. Specificities of primer pairs used in this study

PCR products amplified with primer pair 1 (A–C) and primer pair 2 (D–F) were electrophoresed on 3% agarose gels. Lanes 1–19, the amplification of sugar beet (*Beta vulgaris* L. subsp. *vulgaris*) DNA extracted from seedlings of cultivar Monomidori, Monohikari, Monoperl, Monowhite, Monohomare, Mighty, Schwerdt, Kabutomaru, Yukihinode, Kitamasari, Etopirika, Nozomi, Skane, Freuden, NK-150, C-110, Amano, Hinderupgaad, and Detroit Dark Red, respectively; lanes 20 and 21, the amplification of chard plant (*Beta vulgaris* L. subsp. *vulgaris* var. *cicla*) DNA extracted from seedlings of line FK-02-09 and FK-02-34, respectively; lane 22, the amplification of chenopodium plant (*Chenopodium amaranticolor*) DNA extracted from seedlings; lane 23, the amplification of quinua plant (*C. quinoa*) DNA extracted from seedlings; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. The predicted sizes of the specific amplification products with primer pair 1 and primer pair 2 are 116 bp and 121 bp, respectively.

stranded (ds) DNA using the Qant-iT™ PicoGreen® dsDNA quantitation reagent gave concentrations of extracted DNAs ranging up to 8.36 ng/μL (thin juice), and up to 0.85 ng/μL (brown sugar #2) for in-process samples and commercial products, respectively (Table 1). These results suggested that it was difficult to extract sufficient amounts of DNA at suitable concentrations for PCR analysis from the final products of sugar beets.

Development of sugar beet-specific qualitative PCR method

To detect residual DNA with high sensitivity, we tried to amplify and to sugar beet-specific DNA fragments from the extracted DNAs of the in-process products and the beet sugar products. For this purpose, we designed two pairs of taxon-specific primers, which were elaborated to detect single nucleotide polymorphism (SNP) markers[4]. These primer pairs were specifically able to detect the 19 cultivars of sugar beet investigated in this study (Fig. 2). Amplification was also observed in some reactions with other plants that are closely related with sugar beet, such as chard (*Beta vulgaris* L. subsp. *vulgaris* var. *cicla*), chenopodium (*Chenopodium amaranticolor*), and quinua (*C. quinoa*). No amplification was observed in reactions with DNAs from other plants, *i.e.*, soy, maize, rice, wheat, cotton, oilseed, alfalfa, potato and barley (data not shown). The detection limit of these primer pairs was 5 copies of genomic DNA, calculated from the UV absorption using a C-value of 26 pg/2C[5] (Fig. 3). Therefore, we concluded that these primer pairs were suitable for use in the following experiments. Moreover, these primer pairs would be suitable as taxon-specific controls in future detection methods for GM sugar beets.

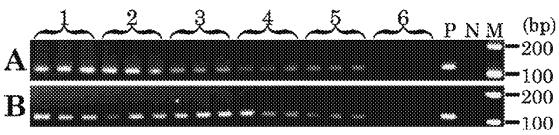

Fig. 3. Estimation of limits of detection (LODs) of sugar beet-specific PCR methods

Successively diluted solutions of genome DNA extracted from leaves of sugar beet plant (cultivar Skane) were prepared and were examined by PCR amplifications with primer pair 1 (A) and primer pair 2 (B). PCR products were electrophoresed on 3% agarose gels. Lanes 1–5, the amplifications of sugar beet DNA extractions containing 500 copies, 100 copies, 20 copies, 10 copies, and 5 copies per reaction, respectively; Lane 6, negative control without template; P, positive control (same as sugar beet genomic DNA samples for 20 copies); M, 100 bp ladder size standard. DNA concentrations were calculated from UV absorption using a *C*-value a 26 pg/2*C*. Each reaction was performed in three replications with independent extractions.

Qualitative PCR analysis of residual DNA in beet sugar

As the template in PCR for in-process samples and beet sugar products, the permissible maximum volume of the undiluted DNA extracts was added to the PCR reactions, and the amount of DNA used for each reaction ranged from 40 ng to 239 ng as calculated from the

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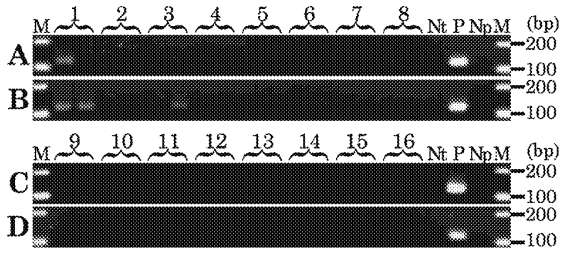

Fig. 4. Analysis of residual DNA in in-process products and commercialized beet sugar product

PCR products amplified with the primer pair 1 (A, C) and the primer pair 2 (B, D) were electrophoresed on 3% agarose gels. Lanes 1–8, detection of sugar beet DNA from the in-process products indicated in Fig. 1; lanes 9–16, detection of sugar beet DNA from commercial beet sugar products, namely brown sugar (1), brown sugar (2), syrup-type product with added oligosaccharide, granulated sugar (1), white superior sugar, brown sugar (3), bleached brown sugar, and granulated sugar (2), respectively. P, detection of sugar beet DNA extracted from seedlings as a positive control; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. Each reaction was performed in two replications with independent extractions.

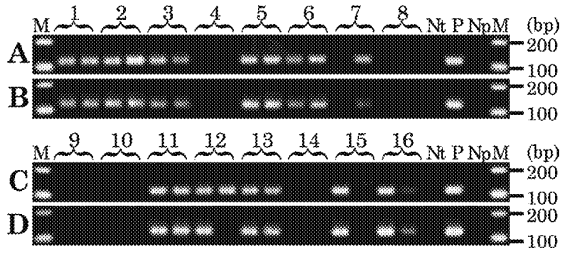

Fig. 5. Inhibition assay

Sugar beet DNA (260 pg) extracted from leaves of sugar beet plant (cv. Skane) was spiked in a reaction mixture containing 16.875 μL of extracts from samples. Amplification was performed with the primer pair 1 (A, C) and the primer pair 2 (B, D), and products were analyzed by electrophoresed on 3% agarose gels after the thermal cycling. Lanes 1–8, detection of sugar beet DNA from in-process products as indicated in Fig. 1; lanes 9–16, detection of sugar beet DNA from commercial beet sugar products, namely brown sugar (1), brown sugar (2), syrup-type product with added oligosaccharide, granulated sugar (1), white superior sugar, brown sugar (3), bleached brown sugar, and granulated sugar (2), respectively. P, detection of sugar beet DNA extracted from leaves as a positive control; Nt, negative control without template; Np, negative control without primers; M, 100 bp ladder size standard. Each reaction was performed in two replications with independent extractions.

UV absorption. In the case of in-process sugars, amplification was observed in the earlier stages of processing, such as thin juice and the thick juice, but no amplification was observed in the case of samples from later stages (Figs. 4A, B). In addition, for inhibition assay of the extracts, we evaluated amplification from samples spiked with 20 copies of sugar beet genome DNA extracted from leaves. The inhibition assays were performed in duplicate for each extract. The results indicated that amplifications were strongly inhibited (2 out of 2) in in-process syrup samples (#4) and brown sugars (#8, #9, #10, and #14) and partially inhibited in in-process syrup samples (#7) and some commercial sugars (#12 and #15) (Fig. 5). Although some samples may contain PCR-inhibitory substances, the results on the in-process samples suggested that the sugar beet DNA was degraded in the early stage of the sugar processing. Thus, it is unlikely residues of DNA are present at measurable levels in commercial beet sugars.

Conclusion

Our results suggested that it is difficult to extract DNA for PCR analyses from processed sugar beets. Although we rely on imported sugar materials, import of raw sugar beets into Japan is forbidden for phytosanitary reasons[*2]. GM sugar beets generally come into the Japanese market as processed sugars or partially purified sugars. In Japan, the mandatory GM labeling is not required for processed foods that do not contain a sufficient amount and/or quality of marker DNAs or proteins, *e.g.* cooking oil and soy sauce[*3]. Based on the result of this investigation, the Japanese government has decided that the mandatory GM labeling is not applicable the sugar products. Moreover, we found taxon-specific primer pairs for sugar beet plants, and clarified that these primers were appropriate to use as taxon-specific controls for GMO analysis by PCR. The results will be useful for future development of detection methods of GM sugar beets.

Acknowledgements

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[*2] http://www.pps.go.jp / english / law / list 1-(20080412-).html

[*3] Notification No. 517 (Mar. 31, 2000), Labeling standard for genetically modified foods. Ministry of Agriculture, Forestry and Fisheries of Japan.

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References

1) James, C. Global status of commercialized biotech/GM crops 2007. ISAAA Briefs, 37, ISAAA, Ithaca, NY (2007).
2) Hino, A. Safety assessment and public concerns for genetically modified food products: the Japanese experience. Toxicol. Pathol., **30**, 126–128 (2002).
3) Yoshimura, T., Kuribara, H., Kodama, T., Yamata, S., Futo, S., Watanabe, S., Aoki, N., Iizuka, T., Akiyama, H., Maitani, T., Naito, S., Hino, A. Comparative studies of the quantification of genetically modified organisms in food from maize and soy using trial producing. J. Agric. Food Chem., **53**, 2060–2069 (2005).
4) Möhring, S., Salaminiand, F., Schneider, K. Multiplexed, linkage group-specific SNP marker sets for rapid genetic mapping and fingerprinting of sugar beet (*Beta vulgaris* L.). Molecular Breeding, **14**, 475–488 (2004).
5) Arumuganathan, K., Earle, E. D. Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep., **9**(3), 208–218 (1991).

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Sugar from genetically modified sugarcane: Tracking transgenes, transgene products and compositional analysis*

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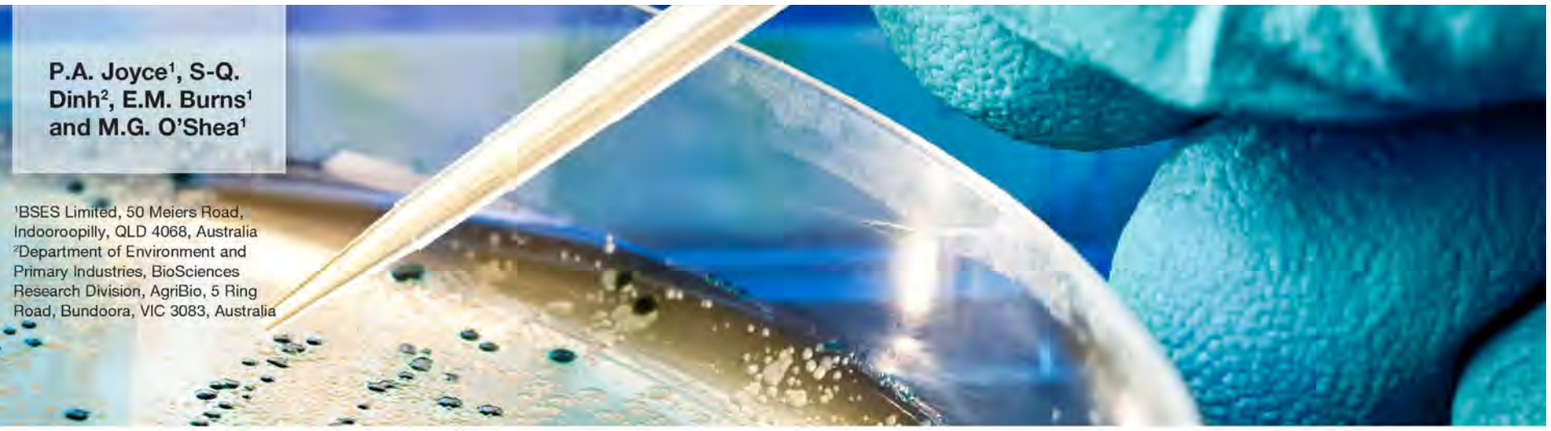

Abstract

This study was performed to prepare the Australian sugar industry for the likely introduction of genetically modified (GM) sugarcane and derived retail sugar products, and to address some of the potential public concerns regarding the characteristics and safety of GM sugarcane products. Juice from representative stalks in a GM field trial of sugarcane plants produced using two different transformation methods (*Agrobacterium*-mediated or biolistics) was compared with untransformed controls [tissue culture (TC) and parent clones (PC)]. The juice was subjected to laboratory scale methods that mimic the factory crystallisation process in order to produce a crystalline raw sugar product. Molecular analysis of the raw sugar sample together with samples collected during each processing step of the laboratory crystallisation process (fibre, juice, syrup, filter mud and molasses) was conducted for the presence of transgenes (DNA) and their products (protein). This testing conclusively showed that although DNA and protein was present in GM sugarcane juice and fibre, it was absent from any samples taken from subsequent processing and crystallisation steps. The sugar compositions of juice and raw sugar produced from GM cane were indistinguishable from those of non-GM cane sourced from the same trial. This study showed that sugar crystallised from GM sugarcane plants does not contain residual DNA or proteins of the introduced transgene(s) using conventional molecular techniques. This finding will help pave the way for commercialisation of GM sugarcane and derived products.

Keywords: ***sugarcane, genetically modified, transgenic, sugar analysis***

Introduction

Transgenic plants are being generated with introduced traits to produce crops with improved yield (James, 2011). This is essential to sustain the increased demand for food as the world population is predicted to reach 10 billion in the next fifty years. Yield improvement is being sought through the incorporation of traits which directly affect plant growth and performance (e.g. improved drought tolerance), value added traits [e.g. 'golden rice' (Potrykus, 2001)] and genes which enhance resistance to pests, diseases or herbicides (e.g. Bt-cotton, papaya resistant to Papaya ringspot virus and RoundupReady® soybean) (James, 2011). Although a number of traits have been introduced to various crops since the early 1990s, only some of these have been commercialised (James, 2011). This is partly due to public concern about the safety of crops possessing 'engineered' genes. Thus, in order for a genetically modified (GM) crop to become successful, this major hurdle of public acceptance needs to be addressed.

The first report of successful transformation of sugarcane appeared in 1992 (Bower and Birch, 1992) when sugarcane callus cells were transformed with reporter genes using a particle inflow gun. Since then, there has been an increase in reports of genetically engineered sugarcane plants using biolistics as well as *Agrobacterium*-mediated transformation methods. Transgenic sugarcane plants with improved disease, pest and herbicide resistance to sugarcane mosaic virus (Joyce *et al.*, 1998), leaf scald (Zhang *et al.*, 1999), stalk borers (Arenciba *et al.*, 1999) and herbicide (Enriquez-Obregon et al., 1998; Manickavasagam *et al.*, 2004) have been produced. Studies on transgenic plants with altered metabolic pathways with a view to improving sucrose accumulation (Botha *et al.*, 2001), other sugar characteristics (Vickers *et al.*, 2005), as well as novel sugars (Basnayake *et al.*, 2012) have also been reported, but none are available commercially.

During the transformation process, a selectable marker gene is generally co-introduced, along with the gene or genes of interest. This is to enable efficient selection of transformed cells on the selective agent to which the marker gene protein confers resistance. Transformed cells have a selective advantage in being able to detoxify this selective agent and thus continue to grow and proliferate on this medium, while untransformed cells are unable to survive. One of the most widely used selectable marker genes is the neomycin phosphotransferase II (*nptII*) gene (Libiakova *et al.*, 2001). This gene and the protein it encodes have been deregulated by the United States Department Agriculture and are considered safe for use in edible GM foods (EPA, 1994). The *nptII* gene occurs naturally in kanamycin-resistant microorganisms found in soil and in mammalian digestive systems (Flavell *et al.*, 1992). The NPTII protein is known to be non-toxic and is rapidly degraded under simulated gastric conditions (Fuchs *et al.*, 1993; Noteborn *et al.*, 1994; Redenbaugh *et al.*, 1994).

The main product derived from sugarcane is crystalline sugar. The production of sugar commences with the extraction of juice from cane stalks in the sugar mill, with the residual stalk fibre (bagasse) reused for co-generation or other products such as particle board. The sucrose-rich juice is clarified by heating (~75°C) and adding lime (calcium oxide-CaO), then filtered leaving behind a thick mud (mill mud).

The clarified juice is concentrated in a series of steps to yield a concentrated syrup from which the sugar crystals are produced. This crystallised raw sugar is removed by centrifugation and the crystallisation process is usually repeated two or three times before the residual syrup (molasses) cannot produce any further sucrose. There are only three reports (two in sugarbeet and one in sugarcane) on the detection of transgenes during sugar crystallisation processes in GM sugar crops.

The two reports in sugarbeet examined the fate of nucleic acid and protein during the sugar manufacturing process (Klein *et al.*, 1998; Oguchi *et al.*, 2009), while the third report in sugarcane was a preliminary study on the detection of DNA in sugar crystallised from GM cane performed in our laboratory (Taylor *et al.*, 1999). Here we report results from a more exhaustive study comparing laboratory crystallised sugar and products of the preceding steps of the milling process in GM sugarcane lines generated using two different transformation methods. All sugar samples were prepared from GM sugarcane for research purposes only, and are not commercially available in Australia.

This work was performed to prepare the Australian sugar industry for the likely future introduction of GM sugarcane and sugar and to respond to some of the inevitable public concern regarding the products of transgenic sugarcane.

Materials and methods

Plant source

GM sugarcane plants were produced from sugarcane variety Q117 using two different transformation methods [*Agrobacterium* (Agro) or biolistics (biol)] using the *nptII* gene as the selectable marker gene, and were grown in the field and harvested at maturity (16 month-old). These transgenic plants were compared with one-eye sett derived parent clones (PC) as well as untransformed tissue culture (TC) controls of Q117. The TC plants were generated using the same protocol as for transformed plants, except that no *nptII* gene was introduced. Representative plant lines (indicated in brackets) from each of the four groups [Agro(3), biol(6), TC(3) and PC(2)] were selected, tagged and leaf samples taken. Juice samples from 16 randomly selected stalks from each of the lines in the four groups were collected and used to crystallise sugar in the laboratory using methods that mimic the factory crystallisation process.

Sampling during the crystallisation process

Two litres of juice were collected from each of the plant lines by crushing the stalks. The fibre after crushing was also sampled. Leaf, juice and fibre samples were stored at –20 °C until required for further analysis. The juice was processed to yield sugar crystals in the laboratory using a protocol and principles similar to that used in the sugar mills (Chen, 1985). Briefly, juice was boiled, filtered and CaO added as a flocculant. The Ca-flocculant was removed by filtration (filter mud) and the pH of the filtered juice was checked and adjusted to >8.5. This clarified juice was concentrated in a rotary evaporator at 70 °C to a syrup containing a brix reading of approximately 80. Raw sugar crystals were produced from the concentrated syrup by seeding with commercial caster sugar and allowing the mixture to crystallise overnight as it slowly cooled to room temperature. Raw sugar crystals were separated from the residual molasses by centrifugation for 10 min at 2000 rpm. Samples were collected at each step of the crystallisation process and analysed for the presence of the *nptII* gene and its product. Samples collected included:

1. Leaf, stalk fibre and raw juice;
2. Syrup and filter mud containing colloidal particles and other residues;
3. Sugar crystals and molasses.

Molecular analyses

Genomic DNA was isolated using the method described by Thomson and Henry (1995) with slight modification for all samples collected at harvest and during each processing step of the laboratory crystallisation process. Briefly, DNA was isolated from 1 mL (solid or liquid sample added to the 1 mL mark of the 2 mL

Table 1. Detection of PCR amplification products specific for *nptII* transgene in leaf, juice, stalk fibre and downstream products of the sugar crystallisation process

	Presence (+) or absence (–) of nptII gene by PCR						
Transformation method	Leaf	Fibre	Juice	Filter mud	Syrup	Sugar crystals	Molasses
Agro	+	+	+	–	–	–	–
Biol	+	+	+	–	–	–	–

Table 2. Determination of NPTII protein concentration (± SE; n = 2–6) by the ELISA test in extracts of leaf and in samples collected at each of the steps during the laboratory sugar crystallisation process

	NPTII protein concentration (ng/mL)						
Transformation method	Leaf	Fibre	Juice	Filter mud	Syrup	Sugar crystals	Molasses
Agro	2.67± 1.77	2.96± 1.18	0.42± 0.21	0	0	0	0
Agro (positive control)	NT	NT	NT	0.24± 0.00	0.17± 0.06	0.27± 0.00	0.18± 0.09
Biol	17.78± 10.00	4.56± 1.75	0.82± 0.36	0	0	0	0
Biol (positive control)	NT	NT	NT	0.25± 0.02	0.2± 0.02	0.22± 0.01	0.13± 0.05

NT – not tested

Table 3. HPLC analyses of soluble sugars in juice and raw sugar crystallised from plants produced by different methods

Sample	Transformation method	Sucrose (g/100 mL)	Glucose (g/100 mL))	Fructose (g/100 mL)
Juice	Agro	23.56	0.10	0.11
	Biol	20.65	0.24	0.26
	TC	21.32	0.28	0.28
	PC	23.88	0.10	0.10
Sugar crystals	Agro	98.00	0.09	0.11
	Biol	97.25	0.19	0.20
	TC	96.30	0.18	0.18
	PC	95.45	0.06	0.04

No significant difference at P≤0.05 between groups for juice or sugar crystal samples

Eppendorf tube) of sample and was homogenised using 400 µL of extraction buffer (100 mM Tris-HCl, 1.0 M KCl, 10 mM EDTA), using the MP FastPrep®-24 Instrument. The extract was centrifuged and the supernatant boiled for 10 min and re-centrifuged. The supernatant was re-extracted using chloroform:isoamyl alcohol (24:1) and the DNA precipitated with isopropanol. The DNA precipitate was washed with 70% ethanol, dried and dissolved in sterile water. This DNA was diluted five-fold and used as the template for the polymerase chain reaction (PCR).

The PCR reaction volume was 20 µL and consisted of 1 µL of DNA added to the reaction mix. *NptII* specific primers (0.5 pmoles each per reaction) were added to a 2× master mix solution (GoTaq Green, Promega) containing Taq polymerase, buffer and dNTPs. The primer sequences for amplification of the *nptII* gene were 5'GAGGCTATTCGGCTATGACTG3' as the forward primer and 5'ATCGGGAGCGGCGATACCGTA3' as the reverse primer. PCR conditions were: initial denaturation for 2 min followed by 35 cycles of denaturation at 95 °C for 15 sec with annealing at 60 °C for 15 sec and extension at 72 °C for 30 sec, and a final extension at 72 °C for 7 min. The presence of the 679bp specific amplification product of *nptII* gene was assessed by gel electrophoresis (results not shown).

Total protein was extracted from each sample using a protein extraction buffer (as per the protocol provided by the manufacturer of the NPTII ELISA kit) and the concentration determined using the Bradford reagent, followed by comparisons with a relative standard curve using bovine serum albumin (BSA). NPTII protein was detected by sandwich ELISA using a commercial NPTII ELISA kit (Agdia), as per the manufacturer's protocol. Absorbance at 450 nm was read in an iMark microtitre plate reader (BioRad).

Sugar analyses

Sucrose, glucose and fructose content of juice as well as raw sugar crystals from all four groups of sugarcane plants were analysed by ion exclusion high pressure liquid chromatography (HPLC) using a Shimadzu HPLC instrument system (Shimadzu Corporation, Kyoto, Japan). Sugar and juice samples were prepared using a 50-fold and 10-fold dilution respectively. Samples (20 µL) were injected onto a Shodex Sugar KS-801 OA column (300 × 8 mm, Phenomenex, Lane Cove, NSW, Australia) operated at 65 °C. The mobile phase was 100% ultrapure water with isocratic elution at a flow rate of 0.9 mL/min. Peaks were identified using a refractive index detector and concentrations were determined by referencing against standard solutions of glucose, fructose and sucrose in the concentration range of 0.05–60 mg/mL. A one-way analysis of variance (ANOVA) was performed on each of the soluble sugars in juice or sugar crystals using the STATISTIX 9 software program.

Results and discussion

PCR analysis showed the presence of the *nptII* gene specific band in leaf, fibre and juice in all the transgenic plants. However, this band was absent in the syrup, filter mud, sugar crystals and molasses indicating that no genomic DNA is carried over during the processing of sugar. Importantly, samples 'spiked' with 10 pg of the *nptII* plasmid DNA showed the presence of this band, indicating that the PCR reaction was not inhibited by the template (Table 1).

Analysis for the presence of NPTII protein in fractions of the sugar crystallisation process showed that the NPTII protein could be detected in leaf, fibre and juice samples. As expected, the concentration of protein in leaf and fibre was greater than that found in the juice samples. No NPTII protein was detected in the filter mud, syrup, sugar and molasses samples (Table 2). When these samples were 'spiked' with NPTII protein (i.e. positive controls), the ELISA tests were positive, indicating that these sample extracts did not interfere with the ELISA reaction.

Positive control samples were spiked with the NPTII protein to demonstrate that the technique was not inhibited by the sample. The concentration of soluble sugars in juice was found to be typical of sugarcane juice samples, with sucrose concentrations between 20–24% and reducing sugar concentrations below 0.3% for both fructose and glucose in all four groups (Table 3).

Analyses of raw sugar crystals also showed no significant differences in relative composition between the four groups and were typical of raw sugar analyses (OECD, 2011). Sucrose concentrations in the crystals were >96% for crystalline sugars from all four groups, with glucose and fructose concentrations below 0.2% (Table 3).

Conclusions

Results presented in this paper show that sugar crystallised from transgenic plants, produced using *Agrobacterium* or biolistic methods, does not contain any detectable residual DNA or proteins of the introduced transgene using conventional molecular techniques. In addition, *nptII* genes could not be detected in syrup, filter mud or molasses. These data agree with those reported in sugar crystallised from sugarbeet (Klein *et al*. 1998; Oguchi *et al*. 2009) where PCR analyses indicated that sugarbeet DNA was degraded at an early stage of the sugar processing. Furthermore, the composition of sucrose, glucose and fructose in juice and raw sugar samples produced from GM cane were indistinguishable from those of non-GM cane sourced from the same trial.

This report on the absence of transgenes and gene products in raw sugar produced from GM sugarcane varieties will assist in gaining public acceptance for the consumption of sugar crystallised from transgenic cane varieties, and will improve the public perceptions surrounding GM sugar and its potential future incorporation within commercial sugarcane production.

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References

Arenciba, A.D., Carmona, E.R., Cornide, M.T., Castiglione, S., O'Relly, J., Chinea, A., Oramas, P. and Sala, F. (1999). Somaclonal variation in insect-resistant sugarcane (*Saccharum* hybrid) plants produced by cell electroporation. *Transgenic Research*, 8: 349 360.

Basnayake, S.W.V., Morgan, T.C., Wu, L. and Birch, R.G. (2012). Field performance of transgenic sugarcane expressing isomaltulose synthase. *Plant Biotechnology Journal*. 10: 217–225

Botha, F.C., Sawyer, B.J.B. and Birch, R.G. (2001). Sucrose metabolism in the culm of transgenic sugarcane with reduced soluble acid invertase activity. *Proc. Int. Soc. Sugar Cane Technol.*, 24: 588–591.

Bower, R. and Birch, R. G. (1992). Transgenic sugarcane plants via microprojectile bombardment. *Plant Journal*, 2:409–416.

Chen, J.P.C. (1985). *Outline of process for manufacturing raw cane sugar*. In: Cane sugar handbook , Part 1:47–55, John Wiley & sons, Inc.

Enriquez-Obregon, G.A., Vazquez-Padron, R.I., Prieto-Samsonov, D.L., Riva, G.A. and Selman-Housein, G. (1998). Herbicide-resistant sugarcane (*Saccharum officinarum* L.) plants by *Agrobacterium*-mediated transformation. *Planta*, 206: 20–27.

EPA (1994). Neomycin phosphotransferase II; tolerance exemption. *Federal Register*, 59: 49351 49353.

Flavell, R.B., Dart, E., Fuchs, R.L. and Fraley, R.T. (1992). Selectable marker genes: safe for plants? *Bio/Technology*, 10: 141–144.

Fuchs, R.L., Ream, J.E.,Hammond, B.G.; Naylor, M.W.; Leimgruber, R.M. and Berberich, S.A. (1993). Safety assessment of the neomycin phosphotransferase II (NPTII) protein. *Nature Biotechnology*, 11: 1543–1547.

James, C. (2011). Global status of commercialised biotech/GM crops: 2011. ISAAA Brief No. 43. ISAAA: Ithaca, NY.

Joyce, P.A., McQualter, R.B., Bernard, M.J. and Smith, G.R. (1998). Engineering for resistance to SCMV in sugarcane. *International Symposium on Biotechnology of Tropical and Subtropical Species* – Part Ii 461: 385–391.

Klein, J., Altenbuchner, J. and Mattes, R. (1998). Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. *Journal of Biotechnology*, 60: 145–153.

Libiakova, G., Jorgensen, B., Palmgren, G., Ulvskov, P. and Johansen, E. (2001). Efficacy of an intron-containing kanamycin resistance gene as a selectable marker in plant transformation. Plant Cell Reports, 20: 610–615

Manickavasagam, M., Ganapathi, A., Anbazhagan, V.R., *et al*. (2004). Agrobacterium-mediated genetic transformation and development of herbicide-resistant sugarcane (Saccharum species hybrids) using axillary buds. *Plant Cell Reports* 23: 134–143.

Noteborn, H., Rienenmann-Ploum, M., van den Berg, J., Alink, G., Zolla, L. and Kuiper, H. (1994). Consuming transgenic food crops: the toxicological and safety aspects of tomato expressing Cry 1Ab and NPTII. In: '*Proceeding of the 6th European Congress on Biotechnology*' Elsevier, Amsterdam.

OECD (Organisation for Economic Cooperation and Development) (2011) Consensus Document on Compositional Considerations for new varieties of sugarcane (*Saccharum* ssp. hybrids): Key food and feed nutients, anti-nutrients and toxicants. Series on the the safety of Novel Foods and Feeds No.23, OECD Environment Directorate, Paris. Available online at the Biotrack website at http://oecd.org/biotrack/

Oguchi, T., Chikagawa, Y., Kodama, T., *et al*. (2009). Investigation of residual DNAs in sugar from sugar beet (Beta vulgaris L.) *Journal of the Food Hygienic Society of Japan*, 50: 42–46.

Potrykus, I. (2001). 'Golden Rice and Beyond'. Plant Physiology, 125: 1157–1161

Redenbaugh, K., Hiatt, W., Martineau, B., Lindemann, J. and Emlay, D. (1994). Aminoglycoside 3'-phosphotransferase II: review of its safety and use in the production of genetically engineered plants. *Food Biotechnology* 8:137–165.

Taylor, G.O., Joyce, P.A., Sedl, J.M. and Smith, G.R. (1999). Laboratory crystallised sugar from genetically engineered sugarcane does not contain transgene DNA. *Proc Aust. Soc. Sugar Cane Technol.*, 21: 502.

Thomson, D. and Henry, R. (1995). Single-step protocol for preparation of plant-tissue for analysis by PCR. *Biotechniques*, 19: 394–400.

Vickers, J.E., Grof ,C.P.L., Bonnett, G.D., Jackson, P.A., Knight, D.P., Roberts, S.E. and Robinson, S.P. (2005). Overexpression of polyphenol oxidase in transgenic sugarcane results in darker juice and raw sugar. *Crop Science*, 45: 354–362.

Zhang, L.H., Xu, J.L. and Birch, R.G. (1999). Engineered detoxification confers resistance against a pathogenic bacterium. *Nature Biotechnology*, 17: 1021–1024.

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Lack of Detection of Bt Sugarcane Cry1Ab and NptII DNA and Proteins in Sugarcane Processing Products Including Raw Sugar

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Brazil is the largest sugarcane producer and the main sugar exporter in the world. The industrial processes applied by Brazilian mills are very efficient in producing highly purified sugar and ethanol. Literature presents evidence of lack of DNA/protein in these products, regardless of the nature of sugarcane used as raw material. Recently CTNBio, the Brazilian biosafety authority, has approved the first biotechnology-derived sugarcane variety for cultivation, event CTC175-A, which expresses the Cry1Ab protein to control the sugarcane borer (*Diatraea saccharalis*). The event also expresses neomycin-phosphotransferase type II (NptII) protein used as selectable marker during the transformation process. Because of the high purity of sugar and ethanol produced from genetically modified sugarcane, these end-products should potentially be classified as "pure substances, chemically defined," by Brazilian Biosafety Law No. 11.105. If this classification is to be adopted, these substances are not considered as "GMO derivatives" and fall out of the scope of Law No. 11.105. In order to assess sugar composition and quality, we evaluate Cry1Ab and NptII expression in several sugarcane tissues and in several fractions from laboratory-scale processing of event CTC175-A for the presence of these heterologous proteins as well as for the presence of traces of recombinant DNA. The results of these studies show that CTC175-A presents high expression of Cry1Ab in leaves and barely detectable expression of heterologous proteins in stalks. We also evaluated the presence of ribulose-1,5-bisphosphate carboxylase/oxygenase protein and DNA in the fractions of the industrial processing of conventional Brazilian sugarcane cultivars. Results from both laboratory and industrial processing were concordant, demonstrating that DNA and protein are not detected in the clarified juice and downstream processed fractions, including ethanol and raw sugar, indicating that protein and DNA are removed and/or degraded during processing. In conclusion, the processing of conventional sugarcane and CTC175-A Bt event results in downstream products with no detectable concentrations of heterologous DNA or new protein. These results help in the classification of sugar and ethanol derived from CTC175-A event as pure, chemically defined substances in Brazil and may relieve regulatory burdens in countries that import Brazilian sugar.

Keywords: sugar, highly purified substance, sugarcane, Cry1Ab, neomycin-phosphotransferase type II

INTRODUCTION

Brazil is the largest sugarcane producer and sugar exporter in the world. With an estimated planted area of 9.1 million ha and a total annual yield of 694.54 million tons of sugarcane, Brazil produces an estimated 39.8 million tons of sugar almost entirely devoted to use as a food ingredient. Ethanol fuel production for the domestic and international markets is also an important use of Brazilian sugarcane, representing half of total annual sugarcane yield. Agricultural biotechnology has been used widely in Brazil for almost 20 years in crops such as soybeans, maize, and cotton and recently the Brazilian biosafety authority CTNBio has approved the first biotechnology-derived sugarcane variety for cultivation.

Sugarcane yield is negatively impacted by pests and diseases typically seen in tropical cultivation conditions. A major insect pest impacting Brazilian sugarcane production is the sugarcane borer (*Diatraea saccharalis*). Infestation by this pest has been shown to reduce shoots, tillers, and plant weight, increase lodging, produce drying of young spindle leaves, and allow infections by opportunistic microorganisms, including bacteria and fungi. Yield losses in excess of 10% and a negative impact on sugar quality (increased levels of secondary metabolites such as dextrans and poor color characteristics) are common as a result of borer infestation (Precetti and Téran, 1983; Precetti et al., 1988; Botelho and Macedo, 2002). Centro de Tecnologia Canavieira (CTC), one of the major suppliers of adapted sugarcane germplasms in Brazil, has developed event CTB141175/01-A (abbreviated here as CTC175-A), which expresses the Cry1Ab protein in leaf tissue to control the sugarcane borer. The event also expresses the neomycin-phosphotransferase type II (NptII) protein used as selectable marker during transformation process. The food/feed and environmental safety of event CTC175-A was extensively evaluated by CTNBio, the Brazilian regulatory authority. Vegetative "seed cane" propagation has begun in controlled field conditions leading to commercial sugar production in Brazil in the 2020 timeframe.

Sugar is extracted from sugarcane stalks which are pressed to produce the sugarcane juice. OECD states that the extracted juice has high water content (about 85%) and contains mainly sucrose and reducing sugars (RSs) like glucose and fructose and that its protein content is negligible, around 0.2% of the dry matter (OECD, 2011). Additionally, sugarcane processing involves harsh conditions known to precipitate and denature protein and DNA, leading to the removal of detectable intact plant DNA and protein in raw and refined sugar (Cullis et al., 2014).

Industrial production of sugar from sugarcane involves extraction of sugarcane juice, clarification, concentration, crystallization, centrifugation, and sugar drying. The sugar processing can be classified as white sugar production and raw sugar production. White sugar can be produced directly from sugarcane if harsh a clarification step is employed. Alternatively, and more usually, white sugar is produced from an additional refining step of raw sugar (Brokensha, 1998). In raw sugar production, juice is physically extracted from the sugarcane by pressing stalks using either a tandem roller mill or diffuser mill. Cut cane pieces are first shredded, immersed in water and then crushed between sets of rollers to release the primary juice (tandem mill); alternatively, shredded cane is extensively rinsed and percolated with recycling ~80°C water to obtain the primary juice (diffuser mill). The residual fibrous material (Bagasse) is typically dried and used as boiler fuel and the surplus is burned to produce electric energy sold to the public grid. In the second phase, primary juice is filtered and clarified by heating at 105°C for 3 h in the presence of lime (calcium hydroxide) and/or a flocculent to precipitate plant macromolecules (protein, DNA, fiber, etc.). The resulting heavy precipitate, called "mud," forms which is separated from the juice in the clarifier, and then filtered to produce filter cake which is removed. The resulting clarified juice (14–20°Brix) is concentrated by vacuum evaporation, at an initial temperature of approximately 110°C which then is decreased to 85–90°C, with concomitant increase of vacuum. This evaporation step finishes when syrup of around 65°Brix is produced (Hugot, 1969; Bruijn, 1998). This syrup is concentrated, at 70°C, in a vacuum evaporative crystallizer to produce raw sugar. The first round of sugar crystallization is performed in around 2–3 h but the process can be repeated several times until no more sucrose crystallizes (Hugot, 1969; Bruijn, 1998). The residual liquid called molasses is mixed with sugarcane juice and yeast and fermented to produce ethanol. After recovery of the ethanol the residual fermentation solids are removed by centrifugation to yield vinasse which is typically used as fertilizer. The next process is the refining of the raw sugar to refined sugar which is the final food ingredient (**Figure 1**).

Therefore, the processes of extraction, raw sugar production, and refining involves multiple steps involving conditions known to denature, precipitate, and eliminate DNA and protein macromolecules found in low concentrations in sugarcane stalks (Cheavegatti-Gianotto et al., 2011; Cullis et al., 2014). As a result, OECD states that sugar is a very purified substance as raw sugar is typically 97–98% sucrose, whereas refined sugar purity is about 99.93% sucrose. The remaining impurities in refined sugar are water, inverted or reducing sugars (glucose and fructose), ash, colored components, and other organic non-sugar compounds (OECD, 2011).

Unlikely other sugarcane producer countries, in Brazil, molasses is almost entirely used for biofuel production, and Brazilian mills do not produce alcoholic beverages, known as "rum," from this residue. A Brazilian sugarcane spirit, known as *cachaça* or *aguardente*, is produced directly from fresh fermented sugarcane juice, in industrial or artisanal facilities which are distinct from sugarcane mills devoted to sugar and ethanol production. In those facilities, after being extracted, the juice is fermented by yeasts to produce the "wine." This wine is then boiled in copper stills giving rise to vapors that are then condensed by cooling producing a liquid with high alcohol content (38–54°GL). The liquid obtained in the initial distillation phase is discarded due to the presence of compounds that are more volatile than ethanol. The last fraction of distillation is also discarded due the presence of low volatile substances. In practice, only the middle fraction of distillation, representing 75–85%, is used for consumption. After distillation, this fraction is filtered and consumed directly or after aged in wood barrels. The vast majority of this cachaça production is devoted to domestic market. The steps of boiling and distilling required for cachaça production are likely to remove traces of protein and DNA from the final product.

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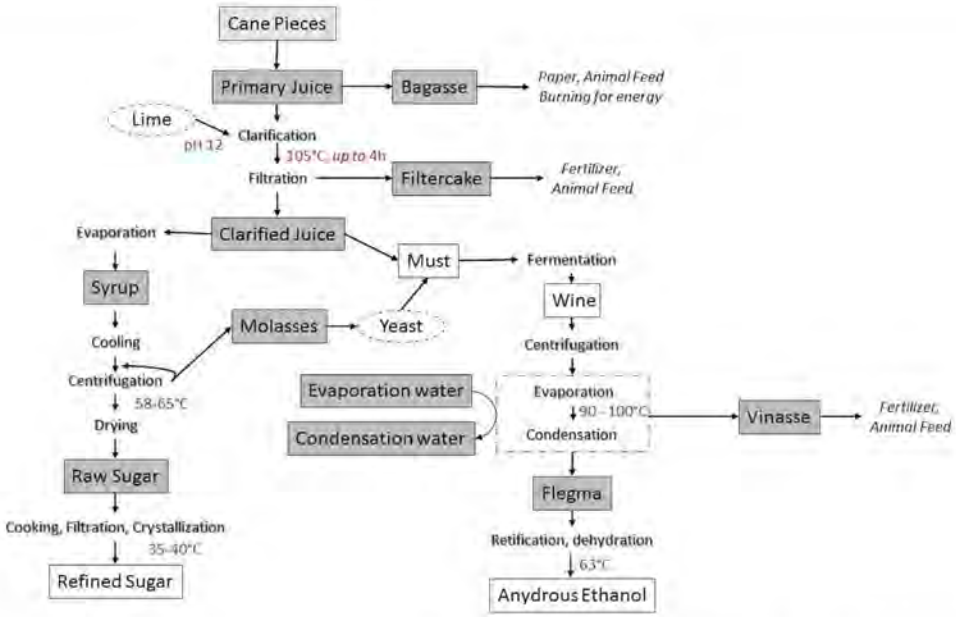

FIGURE 1 | Sugarcane industrial processing and derived food ingredients. Gray boxes indicate sampling points in the process. Lighter gray box (cane pieces) indicates the sampling of leaves.

Due to extremely harsh conditions of sugarcane processing and the resulting purity of those substances, sugar and ethanol produced from all sugarcane, including genetically modified sugarcane, should potentially be classified as "pure substances, chemically defined," by Brazilian Biosafety Law No. 11.105. One of the requirements for this classification is that the substance should not have the GMO itself, neither heterologous protein/DNA in its final composition. If this classification is to be adopted, these substances are not considered as "GMO derivatives" according to Brazilian Biosafety Law. Additionally, this information is important for importer countries to evaluate the food safety of sugar derived from CTC175-A event. The rationale behind the food risk assessment it that the absence, or presence at extremely low levels of heterologous protein in the article of commerce (sugar) would lead to extremely high consumption safety margins due to none/very low exposure to the heterologous protein. By the scientific point of view, this information, in conjunction with the well established safety of Cry1Ab and NptII proteins, should lessen the safety concerns of using sugar derived from CTC175-A as a food ingredient (Kennedy et al., *in this issue*).

Several experiments, described here, were conducted on conventional sugarcane or on event CTC175-A in Brazil. Specifically, studies evaluated the original expression levels of Cry1Ab and NptII in CTC175-A tissues, and the fate of total protein, Cry1Ab and NptII protein and DNA during processing of event CTC175-A sugarcane. Other studies on conventional sugarcane examined the effects of processing on ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), DNA, and protein. Results indicate that CTC175-A expresses heterologous proteins in very low levels at the sugarcane juice, the raw material for sugar and ethanol production, and that sugarcane processing degrades/removes protein and DNA leading to the production of sugar and ethanol in which these substances are not identified by conventional detection techniques.

MATERIALS AND METHODS

Sugarcane Event CTC175-A Expression Cassettes and Newly Expressed Proteins

Event CTC175-A sugarcane was obtained using biolistic plant transformation, by inserting a DNA fragment containing the expression cassettes for the *cry1Ab* and *nptII* genes into sugarcane variety CTC20, a commercially grown conventional variety cultivated in the Center-South region of Brazil. The DNA fragment used in transformation contains the expression cassettes of the *cry1Ab* gene, which encodes a 648-amino acid *Bacillus thuringiensis* protein, and the *nptII* gene, which encodes 263 amino acid type II neomycin phosphotransferase (**Figure 2**).

Cry1Ab is a well-studied insecticidal protein, which confers resistance to certain lepidopteran pests including the sugarcane borer (*D. saccharalis*), while NptII is used as a selectable marker used in the transformation process that confers resistance to aminoglycoside-type antibiotics such as neomycin. The expression of the *cry1Ab* and *nptII* genes is regulated by the promoters of the corn Pepcarboxylase gene (PEPC) and the ubiquitin gene of the corn (ubi-1), respectively. Both genes utilize the nopaline synthase terminator (NOS), from *Agrobacterium tumefaciens*.

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FIGURE 2 | Transformation cassette used to obtain CTC175-A event *via* biolistic transformation of CTC20 sugarcane variety.

The *cry1Ab* gene present in event CTC175-A corresponds to a synthetic and truncated DNA sequence (Koziel et al., 1992). This sequence had its nucleotides synthetically optimized using preferred codons to enhance expression in corn. The *nptII* gene is derived from the Tn5 transposon of *Escherichia coli* (Fraley et al., 1983).

Sugarcane Field Agronomic Management

In order to comply with Normative Resolution No. 05 from CTNBio (*Comissão Técnica Nacional de Biossegurança*—Brazilian Technical Biosafety Commission) which requires evaluation of environmental, food, and feed biosafety, and to analyze its phenotypic performance, the event CTC175-A was planted in six locations representative of the crop area of the progenitor cultivar CTC20 in Brazilian Center-South (Paranavaí—Paraná State, Uberlândia—Minas Gerais State, Montividiu—Goiás State; Conchal, Piracicaba, and Jaboticabal—São Paulo State), in the season 2014/2015.

In each location, standard agronomic practices for sugarcane cultivation (soil preparation, fertilization, pest management) were applied evenly throughout the experiment. Treatments (event CTC175-A and the conventional CTC20) were allocated within each block, forming the plots or experimental units. Each plot was represented by four rows of 10 m spaced by 1.5 m adding up an area of 6.0 m × 10.0 m. The experiments were arranged in a randomized complete block-design with 4 replications. In order to assess Cry1Ab and NptII levels at a time representative of harvest and processing to sugar, tissue samples were collected 365 days after planting in field experiments. All experimental fields were conducted under the official CTNBio approvals obtained through compliance with Normative Resolution No. 06.

Evaluation of Cry1Ab and NptII Expressions in CTC175-A Tissues

In order to evaluate expression levels of proteins Cry1Ab and NptII in CTC175-A event, samples of leaves, stalks, and roots were collected in all replicates from all site experiments and immediately frozen on dry ice until laboratory evaluation. Samples were processed by grinding on dry ice to a fine powder. Protein extractions were performed on representative aliquots of the processed samples. ELISA methodology was used to quantify the proteins in sample extracts.

Cry1Ab protein was extracted from the sugarcane plant tissue samples using the tissue extraction protocol and quantitative assay protocol that follows. An aliquot of each tissue sample was weighed (approximately 15–20 mg) into a 2.0 mL tube. Stainless steel beads were added to each tube. Using buffer ratios of 10:1, an appropriate volume of ELISA extraction buffer (1.5 mL of phosphate-buffered saline with Tween20) was added to each sample. Tissues were pulverized in a Geno Grinder 2010 for approximately 2.5 min at a frequency of 290 *g*. Samples were incubated at 4–8°C for approximately 15 min. Extracts were spun down at ≥12,350 *g* for 10 min at 4°C in a microcentrifuge. Approximately 1.0 mL of the supernatant was collected and placed in a fresh 2.0 mL centrifuge tube. Supernatant from the sample extraction was diluted in deionized water to fall within the range of the standard curve. Remaining supernatants were then frozen at −20°C. The presence of the Cry1Ab protein was detected using a validated ELISA (EnviroLogix Qualiplate Cry1Ab ELISA kit).

Neomycin-phosphotransferase type II protein was extracted from the sugarcane plant tissue samples using the tissue extraction protocol and quantitative assay protocol that follows. An aliquot of each tissue sample was weighed (approximately 45–55 mg for leaf tissue and 190–210 mg for root and internode tissue) into a 2.0 mL tube. Four stainless steel beads were added to each tube. An appropriate volume of ELISA extraction buffer was added to each sample. 1.5 mL of 1× PEB (supplied with kit) was used. Tissues were pulverized in a Geno Grinder 2010 for approximately 2.5 min at a frequency of 290 *g*. Samples were incubated at 4–8°C for approximately 15 min. Extracts were spun down at 12,350 *g* for 10 min at 4°C in a microcentrifuge. Approximately 1.0 mL of the supernatant was collected and placed in a fresh 2.0 mL centrifuge tube. Supernatant from the sample extraction was diluted in 1× PEB to fall within the range of the standard curve. Remaining supernatants were then frozen at −20°C. The presence of the NptII protein was detected using a validated ELISA assay (Agdia NptII ELISA Kit).

Control sample extracts were analyzed concurrently to confirm the absence of plant-matrix effects in ELISA. For each ELISA, a standard curve was generated with known amounts of the corresponding reference protein. Cry1Ab protein standard calibrators at 100, 75, 50, 25, 12.5, 6.25, 3.13, and 0 ng/mL were prepared in deionized water. NptII protein standard calibrators at 20, 15, 10, 5, 2.5, 1.25, 0.625, and 0 ng/mL were prepared in PBST. Calibrators were prepared fresh each day from a working stock solution. The mean absorbance for each sample extract was plotted against the appropriate standard curve to obtain the amount of protein as nanograms per milliliter (ng/mL) of extract. The concentrations were converted to represent the amount of protein as micrograms per gram (μg/g) of tissue by the following formula:

$$(\text{ng/mL}) \times (\text{dilution factor}) \times (\text{volume of buffer [mL]}) / (\text{amount of tissue[g]}) \times 1{,}000.$$

The predetermined extraction efficiencies were used to adjust the transgenic protein concentrations to the estimated total

concentration in the corresponding tissue sample by the following formula:

$$\text{amount of protein measured from a single extraction } (\mu g/g) / \text{extraction efficiency } (\%).$$

All calculations, including mean and SD, were performed with Microsoft Excel® 2007 spreadsheet software. All decimal places associated with the concentrations determined for each replicate sample were used in calculation of the mean, where were then rounded to two decimal places for reporting consistency.

CTC175-A and CTC20-Derived Sugar and Ethanol Production at Laboratory Scale to Evaluate DNA and Protein Loss

In order to comply with Brazilian Biosafety Normatives for regulated genetically modified plant material, one batch of sugar from CTC175-A and one batch of sugar from CTC20 were produced. Mature stalks of CTC175-A event sugarcane and the parental conventional variety CTC20 were collected from all plots of the experiment planted at Piracicaba/SP at 365 days after planting and processed into raw sugar and ethanol using laboratory scale methods (Novello, 2015; Merheb, 2014; Merheb et al., 2016) that mimic the industrial processes used by Brazilian mills. Harvested stalks were immediately transported to the laboratory for sugarcane juice extraction and subsequent processing to collect process fractions including raw sugar.

Approximately 56.0 L of sugarcane juice was extracted from 90 stalks of each variety by shredding and pressing in the laboratory. Leaf, fiber, and sugarcane juice samples were collected from each variety for DNA, protein, and sugar quality analyses. The stalk quality of sugarcane varieties was evaluated by analyses of fiber, starch, brix, dextran, RSs, total RSs, pH, polarization, and purity (**Table 1**). These characteristics are factors that have a direct impact on the quality of the final products and the yields of the processes (Santos et al., 2012).

The sugarcane juices of CTC175-A and CTC20 were heated to 70°C with constant stirring, immediately after reaching 70°C, the juice was neutralized (pH 7) by adding lime. Following neutralization, juice was further heated to 98–100°C for approximately 2 min, and then transferred to a vessel, containing approximately 3 ppm of anionic polymer flocculant (Flonex 9076)/liter of juice. Following flocculation and decantation, clarified juice (supernatant) was separated from the sludge and samples were collected.

Clarified juice was concentrated from 20 to 65° Brix to generate syrup, using a rotary evaporator. After concentration, this syrup was used in crystallization which was performed using a laboratory reactor (Marconi MA 502), with an 8.0 L internal volume, that was equipped with a helical-type agitator. After the preparation of syrup, 1.0 L of syrup was added in crystallizer to be concentrated from 65 to 84° Brix in vacuum (22in Hg). At this point, 30 g of refined sugar were seeded. Afterward, in the same vacuum, the crystallizer feeding was performed by a controller. When feeding stopped, the crystallizer was in standby for 90 min, and the final evaporation started to be concentrated from 84 to 90° Brix in vacuum. After 6 h, vacuum was removed and the mass was centrifuged and washed with steam. The resultant dense mass of sugar crystals was centrifuged using a laboratory basket centrifuge (Metalúrgica Sueg Ltda), with a capacity of 1.0 kg of crystal sugar per batch (Merheb, 2014; Merheb et al., 2016). In these experiments, approximately 1.0 kg of sugar was produced per crystallization. Following centrifugation, sugar crystals were air-dried for approximately 12 h (Merheb et al., 2016).

TABLE 1 | Methodologies used for analyzing characteristics used for sugar classification in Brazilian market.

Characteristic	Methodology	Reference
Starch	Starch—determination in raw sugar	ICUMSA Method GS 1-16 (2009)
Ash	The determination of conductivity ash in raw sugar, brown sugar, juice, syrup, and molasses	ICUMSA – Método GS 1/3/4/7/8-13 (1994)
Color	Determination of solution color of raw sugars brown sugars and colored syrups at pH 7.0	ICUMSA – Method GS 9/1/2/3-8 (2011c)
Dextran	The determination of dextran in raw sugar by a modified alcohol haze method	ICUMSA Method GS 1/2/9-15 (2011b)
Filterability	Método BR-SM-PR-103	Supplemental Methodology S1
Acid Floc	Método BR-SM-PR-420	Supplemental Methodology S1
Alcohol Floc	Método BR-SM-PR-271	Supplemental Methodology S1
RS	Method 32—reducing sugars—determination in raw sugar by the Lane and Eynon method	The Laboratory Manual for Australian Sugar Mills (2001)
Polarization	The determination of the polarization of raw sugar by polarimetry	ICUMSA GS 1/2/3/9-1 (2011a)
Turbidity	Methods of analysis—formazin turbidity standards	ASBC (1976)
Sugars	High-performance liquid chromatography (HPLC)	Sluiter et al. (2006)

Vinasse and Flegma (diluted ethanol) were obtained from the juice in a single cycle batch fermentation performed in triplicate using must composed of sugarcane juice with approximately 160 g/L TRS and 100 g/L fresh PE-2 industrial yeast in a final fermentation volume of 500 mL. Fermentation was performed in an Erlenmeyer flask placed in a shaker (Innova 44, New Brunswick Scientific) at 0.805 *g* and 32°C for 8h. Simple distillation was performed to separate flegma resulting from fermentation from the vinasse, using a distiller (Tecnal Redutec TE–086 alcohol microdistiller). The wine was heated to 90–100°C for 5 min for flegma evaporation and condensation. Vinasse was the distillation residue. For ethanol production, it is necessary to use a distillation column to purify the flegma into ethanol.

All sugar production and sugar analysis were performed in laboratories certified with CQB (*Certificado de Qualidade em* Biossegurança—Biosafety Quality Certification) granted by CTNBio according to Normative Resolution No. 01. All personnel working in these activities were trained according the requirements of Normative Resolution No. 02 for contained activities with genetically modified plant material.

Compositional Analysis of Sugar Produced in Laboratory

Raw sugars and other common parameters were analyzed at CTC's laboratories certified with CQB to comply with Brazilian biosafety requirements. The quality of the raw sugar produced from either event CTC175-A or CTC20 conventional was assessed for sugar quality parameters: starch, ash, color, dextran, filterability, acid floc, alcohol floc, RSs, polarization, turbidity, and sugars (sucrose, glucose, fructose). The analytical methodologies used to classify sugar according to Brazilian market are presented in **Table 1** and are relevant for sugar classification and placement to specific markets in Brazil (Oliveira et al., 2007a,b).

DNA Detection in Laboratory Sugar Fractions

To evaluate the fate of DNA and proteins at the different laboratory processing stages, samples were collected during the processing of sugarcane to raw sugar and ethanol. Both solid samples (leaf, bagasse, and sugar) and liquid samples (primary juice, clarified juice, sludge, syrup, molasses, flegma, and vinasse), were collected from both cultivars (CTC175-A event and CTC20 isoline). The DNA extraction protocol used was based on Aljanabi et al. (1999) with modifications. Solid samples were ground in liquid N_2 and 5.0 mL of samples were added to 4.0 mL of homogenization buffer (200 mM Tris–HCl, 50 mM EDTA, 2.2 M NaCl, 2% CTAB, 0.06% Na_2SO_3, pH 8.0). A detergent solution (2.0 mL of 5% N-lauryl-sarcosine, 2.0 mL of 10% PVP, 2.0 mL of 20% CTAB) was added to the homogenized samples and mixed by inversion for 2–3 min, then incubated for 60 min at 65°C with periodic inversions. After incubation, 10.0 mL of 25:24:1 phenol: chloroform: isoamyl alcohol were added to the samples and mixed by inversion for 2 min. After centrifugation (1,520 *g*, 10 min, 4°C), the supernatant was transferred to a new tube, 10.0 mL of 24:1 chloroform: isoamyl alcohol was added, and mixed by inversion for 2 min. After centrifugation (1,520 *g*, 10 min, 4°C), the supernatant was transferred to fresh tubes, 10.0 mL of isopropanol and 2.0 mL of 6 M NaCl were added, and the tubes were mixed by inversion for 2 min. The samples were then incubated (20°C, 1 h), centrifuged (1,520 *g*, 5 min, 4°C), and the formed pellets were washed two times with 10.0 mL of 70% ethanol. The pellets were dried at room temperature and dissolved in 200 µL of sterile ultrapure water. All DNA samples were quantified in a NanoDrop™ 8000 spectrophotometer (ThermoFisher™). In addition to the total DNA quantification results, all samples from both cultivars (CTC20 and CTC175-A), were also evaluated for the presence of heterologous DNA representing *cry1ab* (GenBank Accession No. AY326434.1) and *nptII* (GenBank Accession No. U00004) genes and the endogenous *ubi1* gene (GenBank Accession No. CA179923.1) genetic elements by TaqMan multiplex analysis (**Table 2**).

The TaqMan® Multiplex Assay protocol for DNA detection was performed as follows: the reactions were performed in multiplex form to amplify simultaneously one of the two combinations: *cry1Ab/ubi1* or *nptII/ubi1*. PCR was performed in a 96-well optical plate ("MicroAmp® Fast Optical 96-Well Reaction Plate™," Life Technologies). The performance of the assays in each processing sample type (e.g., primary juice, bagasse, etc.) was assessed by adding known quantities of the specific DNA for *cry1ab*, *nptII*, and *ubi1* to processing fractions produced from the CTC20 isoline. All samples were normalized for final DNA concentration of 10 ng/µL and 40 ng were used for each PCR. For samples with DNA concentrations below limit of detection (LOD), 4 µL of DNA solution was used. For positive control samples, two known concentrations of each target gene were used (0.5 and 0.05 ng of DNA).

Reactions were assembled using samples added to a mixture of the following reaction components: 1× Taqman® II Mix Universal Buffer UNG (Applied Biosystems™); forward and reverse primers, each at 500 nM concentration; probes at a final concentration of 200 nM for the multiplex assay cry1Ab/ubi; forward and reverse primer, each at 300 nM concentration; probes at a final concentration of 200 nM for the multiplex assay nptII/ubi; and water in sufficient quantity to make up a final volume of 20 µL. Plates were sealed with optical adhesive film for real-time PCR MicroAmp® (Applied Biosystems™) and PCR was performed in a thermocycler 7500 Fast Real-Time PCR System (Applied Biosystems™) using the following amplification parameters: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primers and probes sequences used are detailed in **Table 2**.

Each PCR amplification curve was examined to determine the presence (+) or absence (−) of DNA by comparing with the respective amplification curve of the positive control. The control DNA concentrations (0.5 and 0.05 ng), were chosen because they represent reliable detection limits of the methodology. Samples that had amplification (Cq) values higher than the positive control at the lowest concentration of DNA (0.05 ng), were considered as non-specific amplifications (<LOD).

Detection of Total Proteins in Laboratory Production Fractions

For the extraction and quantification of total proteins, an aliquot (500 µL) of samples taken from the laboratory production of sugar (i.e., leaf, bagasse, juice, filter cake, clarified juice, syrup, molasses, sugar, phlegm, and vinasse) was mixed with 750 µL of protein extraction buffer (0.01 M phosphate buffered saline: 0.138 M NaCl; 0.0027 M KCl, 0.05% TWEEN® 20, pH 7.4), homogenized and centrifuged (10 min, 7.690 *g*). After centrifugation, 600 µL of supernatant was transferred to new tubes and the

TABLE 2 | Primers and probes sequences used to identify exogenous (*cry1Ab* and *nptII*) and endogenous (*ubi*) genes present in CTC175-A by qPCR.

Target	PCR product (pb)	Primer (5′ → 3′)	Primer (5′ → 3′)	Probe
cry1ab	102	GTGGACAGCCTGGACGAGAT	GAAGCCACTGCGGAACATG	CCCCTCAGAACAAC
nptII	103	GCTCACCCTGTTGTTTGGTGTT	AGCCTCTCCACCCAAGCG	CTTCTGCAGGTCGACTC
ubi1	63	ACCATTACCCTGGAGGTTGAGA	GTCCTGGATCTTCGCCTTCA	CTCTGACACCATCGAC

samples were analyzed for total protein concentration using the Bradford method. A seven-point standard curve with concentrations ranging from 125 to 2,000 mg/mL of bovine serum albumin was produced.

A 96-well flat-bottomed plate was assembled using 10 µL of buffer (null control), 10 µL of each standard (in duplicate) and 10 µL of each sample studied. Bradford solution (200 µL) was added (Bio-Rad Protein Assay Dye Reagent Concentrate™) to each well in a 1:4 ratio (dye:water). The plate incubated on the bench for 5 min and was read on a M2-SpectraMax spectrophotometer (Molecular Devices™).

Detection of Cry1Ab and NptII Proteins in Laboratory-Processed Samples

All samples were analyzed for Cry1Ab protein presence using the "QualiPlate™ ELISA Kit for Cry1Ab/Cry1Ac" (ENVIROLOGIX ™) according to the manufacturer's recommendations. Data were generated using the SpectraMax-M2 Spectrophotometer (Molecular Devices™). A known amount of Cry1Ab protein (~ 0.50 ng) was used to spike a portion of the samples obtained during the production of sugar and ethanol from CTC20-derived samples to obtain an expected final concentration of approximately 3.0 ng/mL of Cry1Ab protein for each sample. A serial dilution curve of the Cry1Ab protein was positioned adjacent to investigated samples in the plate to determine the LOD of the assay. The dilution curve ranged from 50 ng to 3.125 ng/mL of protein.

Collection of Conventional Sugar Samples From Brazilian Sugarcane

Samples from the industrial processing of conventional sugarcane were collected at two different types of industrial sugarcane mills in Brazil: a tandem roller type ("mill F") and a diffuser type ("mill C"). Nine sample types were collected from each mill, in the harvest 2016/17: bagasse, primary juice, filter cake, clarified juice, syrup, molasses, vinasse, raw sugar, and flegma. Leaves from CTC20 variety were used as a control sample that does not contain the heterologous DNA and newly expressed proteins. All samples were transported on ice (2–4°C) and stored at −80°C. Leaves from CTC20 variety were used as a control. All samples were stored and transported to CTC on blue ice (2–4°C), and immediately frozen after arriving.

Total and RuBisCO DNA Detection in Processing Fractions Obtained From Brazilian Sugarcane Processing Mills

DNA of each fraction was isolated (5.0 mL wet or dry samples in a 50.0 mL conical tube) following the DNA extraction protocol described by Aljanabi et al. (1999). DNA samples were concentrated in an Eppendorf™ Vacufuge™ to a final volume of 0.2 mL before quantification in a Qubit® fluorometer (LifeTechnologies) following the protocol suggested by the manufacturer. DNA samples were also assessed for quality by visualization on ethidium bromide-stained agarose gels.

The sequence of the RuBisCO large subunit of *Saccharum* hybrid cultivar SP80-3280 (GenBank: AE009947.2) was used to design primers for PCR assays (*Saccharum* hybrid cultivar SP-80-3280 chloroplast, complete genome: 119082-120512). Primers were designed to specifically amplify fragments of different sizes. The sequences of the primers and the expected fragment sizes are given in **Table 3**.

A dilution curve was prepared using total DNA from sugarcane leaves. Decreasing concentrations ranged from 50 to 0.0125 ng. Reactions were prepared in a final volume of 25 µL using the following reaction components: 1× DreamTaq Green PCR Master Mix (Thermo Scientific), 0.2 µM of each primer, and 5 µL of genomic DNA of known concentrations, to have five different points of dilution curve for each tested pair of primers (50, 25, 12.5, 0.125, and 0.0125 ng).

PCRs were performed on the Proflex® thermal cycler (Applied Biosystem), according to the following step-cycle program: initial denaturation step at 92°C for 2 min; 30 cycles consisting of denaturation at 92°C for 30 s, annealing at 60° for 40 s (primers combinations 1, 2, and 3) or annealing at 50°C (primers combinations 4 and 5), and extension at 72°C for 60 s; final extension step at 72°C for 7 min. After the amplification, PCR products were electrophoresed on 2% agarose gels in 1× TBE solution, stained with 0.4 µg/mL ethidium bromide, visualized under ultraviolet light (UV), and registered with transilluminator and software L.PIX Loccus Biotechnology.

Reactions were performed in 25 µL final volume with variable amounts of template. For fractions from which DNA was quantifiable, serial dilutions were prepared; seven points (10, 5, 0.5, 0.25, 0.025, 0.0025, and 0.00125 ng) were amplified in each PCR. When DNA concentrations from fractions were below the LOD (<LOD), an arbitrary volume of sample was added to the amplification reactions. Thus, for raw sugar and flegma from the tandem roller mill samples and, raw sugar, flegma, and clarified juice samples from diffuser mill 5, 2.5, 1.25, and 0.5 µL of template were used. DNA from leaf was used as positive control (2.5 ng). In parallel, an aliquot of the same matrix of each sample was spiked with DNA from sugarcane leaves. Again, a serial dilution was done to have an input of approximately 2, 1, 0.1, 0.01, and 0.001 ng of DNA in each reaction. For raw sugar and flegma from both types of mills and for clarified juice from diffuser mill, the dilution curve consisted of 12.5, 6, 3, and 1 pg were used. Reaction components and the cycling program followed as described previously, using 28 amplification cycles. PCR products were electrophoresed on 2% agarose gels.

Total Protein Detection in Processing Fractions Obtained From Brazilian Sugarcane Processing Mills

After freezing, 6.0 mL of each sample was aliquoted in six tubes (1.0 mL each) and lyophilized for 6 days at −60°C with exception

TABLE 3 | Primers name, primers sequences, and expected size of amplified fragments.

Combinations	Primer name	Sequence 5′ → 3′	bp
1	SoRcbL_TqM.F SoRcbL_R.1	CGCCTCACGGTATCCAAGTT CGGTTTCGGCTTGTGCTT	246
2	SoRcbL_TqM.F SoRcbL_R.2	CGCCTCACGGTATCCAAGTT TGCTCGGTGAATGTGAAGAAG	437
3	SoRcbL_F SoRcbL_R.2	CGGAGTACGAAACCAAGGATAC TGCTCGGTGAATGTGAAGAAG	809

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of flegma. Samples from leaves and bagasse were further ground to a homogeneous powder and protein extraction was performed as described by Cullis et al. (2014) with minor modifications. Lyophilized samples from individual tubes combined into a 15-mL polypropylene tube and prepared as solutions, where former solid samples (leaves, bagasse, and filter cake) were dissolved at a 3% (w/v) in water. Similarly, primary juice, clarified juice, syrup, molasses, raw sugar, and vinasse were dissolved in a 10% (w/v) in water. Flegma was prepared as a solution 20% (v/v) in water. This procedure was done in duplicate, with half of the mill's fractions (controls) spiked with 1,000 ng of total protein, previously extracted from sugarcane leaves and quantified using microplate "Micro BCA protein assay" (ThermoFisher). In the case of raw sugar and flegma, aliquots were spiked with 10 µg of total protein. All solutions were adjusted to 1% sodium dodecyl sulfate (SDS) + 10 mM dithiothreitol + 10 mM Tris–HCl pH 7.5 + 0.5 mM PMSF (SDS extraction buffer) and placed at 65°C for 60 min with occasional mixing by inversion. Tubes were centrifuged (6,500 *g*) for 15 min at room temperature. To 2 mL of the supernatant, 3 mL of 1% sodium deoxycholate were added followed by 1.25 mL of 50% trichloroacetic acid. After mixing and incubating on ice for 15 min, the tubes were centrifuged at 6,500 *g* for 15 min at 4°C, supernatant was discarded, and the pellet drained for 5 min. Pellets were then washed with 1.5 mL of acetone by vigorous mixing for 15 s followed by incubation at 25°C for 15 min with occasional mixing. Samples were placed on ice for 10 min, centrifuged, the supernatants removed, and the tubes drained at room temperature. Next, 1.5 mL of 85% acetone was added with mixing, and the tubes were centrifuged, drained, and dried at 37°C for 15 min. The precipitate was dissolved in 0.5 mL of 0.5% SDS + 10 mM Tris–HCl pH 7.5 at 65°C for 20 min, with occasional mixing. Assuming 100% of recovery, the concentration of total proteins in spiked samples per microliter of resuspended extract, should be 2 ng/µL. Protein content was determined in both, original mill fractions and spiked mill fractions (controls), using the microplate Micro BCA protein assay (ThermoFisher) as recommended by the manufacturer. SDS-PAGE was used to check extracted protein quality of each sample. About 2–20 µg of total protein were diluted in sample buffer (2× Laemmli Buffer, Biorad, USA) and denatured at 100°C for 5 min. Proteins were separated under denaturing conditions on a 4–20% polyacrylamide gel (Mini-PROTEAN TGX, Biorad, USA) ready to use. At the end, protein gels were stained with Coomassie Brilliant Blue (EZBlue, Sigma, USA).

RuBisCO Protein Detection

ELISA was performed according the manufacturer's recommendations ("Plant RuBisCO ELISA Kit"—Catalog # MBS705973—MyBioSource). The detection range described in the kit's protocol is 3.12–800.0 µg/mL. Thus, using the standard sample solution supplied with the commercial kit a standard curve was prepared with five points of known RuBisCO concentration (µg/mL). The protein sample eluent (0.5% SDS + 10 mM Tris–HCl) serves as the zero standard and the curve blank. The total protein samples and the buffer used to elute the protein after extraction were diluted to 1/2, 1/5, 1/10, and 1/100. The mean absorbance of each buffer dilutions was used as a blank for each sample dilution. The concentration read from the standard curve was multiplied by the dilution factor. The optical density of each well was determined using a microplate reader (SpectraMax, Molecular Devices, USA) set to 450 nm with wavelength correction of 540 and 570 nm.

RESULTS AND DISCUSSION

Expression of Cry1Ab and NptII on Tissues of CTC175-A Events

The construct used to obtain CTC175-A event was designed to express Cry1Ab preferentially in leaves, where the sugarcane borer lays its eggs and starts its development. The promoter used to drive Cry1Ab expression, PEPC, is known to confer preferential expression in photosynthesizing tissues (Harrison et al., 2011). Therefore, as expected, the highest concentrations of Cry1Ab were found in leaf tissue in all evaluated sites; much lower levels were found in roots and stalks [below the limit of quantification (LOQ) of ≤235 ng/g FW tissue] (**Table 4**).

In **Table 4**, Cry1Ab expression values in leaves were expressed as mean of four repeats, with their respective SE and SDs, for each site. As for root and stalk tissues, expression of Cry1Ab is much lower and some repeats data were below of LOQ of ELISA assay. When at least one repeat had measurement above LOQ, data of Cry1Ab expression for those repeats above LOQ were reported directly without any statistical analysis. It was only possible to detect Cry1Ab expression in stalks, the raw material for sugar and ethanol production, for only one repeat of each site, Conchal (310 ng/g FW tissue) and Paranavaí (370 ng/g FW tissue).

The expression of NptII on event CTC175-A was solely required for selecting transformed events during the transformation

TABLE 4 | Concentration of Cry1Ab protein in tissues of CTC175-A event evaluated *via* ELISA ($n = 4$).

Site	Tissue	Cry1Ab (µg/g FW)	SD	SE
Jaboticabal	Stalk	<LOQ	–	–
	Leaf	64.01	9.59	4.8
	Root	0.40/0.55/0.58[a]	–	–
Montividiu	Stalk	<LOQ	–	–
	Leaf	40.7	10.5	5.2
	Root	0.81/0.83/0.87[a]	–	–
Piracicaba	Stalk	<LOQ	–	–
	Leaf	64.9	11	5.5
	Root	<LOQ	–	–
Conchal	Stalk	0.31[b]	–	–
	Leaf	63.4	6.7	3.4
	Root	0.67/0.70[c]	–	–
Uberlândia	Stalk	<LOQ	–	–
	Leaf	67.9	20.6	10.3
	Root	<LOQ	–	–
Paranavaí	Stalk	0.37[b]	–	–
	Leaf	29.6	3.1	1.6
	Root	0.84[b]	–	–

Limit of quantification for leaf, stalk, and root tissues: LOQ ≤ 0.235 µg/g.
[a]*Three repeats above LOQ.*
[b]*One repeat above LOQ.*
[c]*Two repeats above LOQ.*

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process. The *Ubi* promoter driving expression of the *nptII* gene in the transformation cassette is known to be expressed in rapidly dividing tissues (Christensen et al., 1992). Therefore, results show low levels of NptII expression in leaves 0.07–0.16 µg/g FW and even lower expression in roots ranging from below the LOQ (<34 ng/g FW) to 70 ng/g FW. NptII expression was at or below detectable levels in sugarcane stalks (<34 ng/g FW) (**Table 5**).

At **Table 5**, as for Cry1Ab, NptII expression values in leaves were expressed as mean of four repeats, with their respective SEs and SDs, for each site. As for root and stalk tissues, expression of NptII is much lower and some repeats data were below LOQ. When at least one repeat had measurement above LOQ, it was decided to report data of NptII expression for those repeats above LOQ, without any statistical analysis.

These results indicate that sugarcane stalks, which are the raw material for sugar and ethanol production, presents originally low levels of heterologous protein expression. This is not surprising due to the nature of promoters used to drive gene expressions and the fact that sugarcane naturally presents negligible protein levels in stalks (OECD, 2011). In fact, the search for promoters that ensures high protein expression levels in sugarcane stalks is still a scientific challenge (Damaj et al., 2010).

Composition of Sugar Obtained From the Laboratory Processing

The quality of the harvested sugarcane for industrial processing is an important consideration for processing mills because stalk quality directly affects the sugar and ethanol production potential (Garcia, 2012; Santos et al., 2012; Santos and Borem, 2013). One batch of sugarcane juice for CTC175-A event and one for CTC20 cultivar were prepared, therefore, the values presented at **Tables 6** and **7** should be evaluated as single measures and not as estimates of quality parameters of juices and sugars produced from CTC175-A and CTC20. Despite this, the results of quality parameters of both juices were within the recommended range for these sugarcane analytes (**Table 6**), confirming that the raw material used for the laboratory production for sugar and ethanol in this study was acceptable. This procedure is commonly used by Brazilian mills to evaluate and to pay according to the content of sucrose (Pol% juice) in sugarcane (Bruijn, 1998). The other parameters were evaluated for key sugarcane processing steps. Overall, juices produced in laboratory scale resembled juice ordinally processed in Brazilian mills.

In Brazil, it is the usual practice to employ COPERSUCAR specifications to classify sugars for different industrial applications. According to the physicochemical parameters evaluated (**Table 7**), sugar produced from both the event CTC175-A and CTC20 conventional were classified as "Type 3C" according to the classifying parameters: conductometric ashes $\leq$ 0.1%, color ICUMSA $\leq$ 400 and sugar content (Pol Z) above 99.5%, published by COPERSUCAR (2015). The high quality of Type 3C sugar produced, which technically can be labeled as "white sugar," is not surprising even though the sugar production method employed here was the typical method for production "raw sugar." This higher grade result can also be obtained in real world sugar production when the quality of starting sugarcane juice is high (Santos et al., 2012).

TABLE 5 | Concentration of NptII protein in tissues of CTC175-A event evaluated *via* ELISA ($n = 4$).

Site	Tissue	NptII (µg/g FW)	SD	SE
Jaboticabal	Stalk	<LOQ	–	–
	Leaf	0.15	0.02	0.01
	Root	<LOQ	–	–
Montividiu	Stalk	0.02[a]	–	–
	Leaf	0.08	0.02	0.01
	Root	0.04/0.04[b]	–	–
Piracicaba	Stalk	0.01[a]	–	–
	Leaf	0.16	0.02	0.01
	Root	0.07	0.01	0.01
Conchal	Stalk	0.02[a]	–	–
	Leaf	0.13	0.03	0.02
	Root	0.05	0.01	0.01
Uberlândia	Stalk	0.01[a]	–	–
	Leaf	0.03	0.07	0.03
	Root	0.04[a]	–	–
Paranavaí	Stalk	0.02/0.01[b]	–	–
	Leaf	0.13	0.05	0.02
	Root	0.06/0.24[b]	–	–

Limit of quantification for leaf tissues: LOQ ≤ 0.034 µg/g. Limit of quantification for stalk and root tissues: LOQ ≤ 0.0094 µg/g.
[a]*One repeat above LOQ.*
[b]*Two repeats above LOQ.*

TABLE 6 | Results of sugarcane quality analysis.

Characteristic	Unit	Recommended values (Santos and Borem, 2013)	CTC20	CTC175-A
Fiber	% sugarcane	10–13	10.72	10.41
Starch	mg/kg	<1,000	875	948
Brix	% juice	>14	21.02	20.84
Dextran	mg/kg	<10	<10	<10
RS	% juice	<0.8	0.47	0.47
TRS	% juice	>15	20.54	20
pH	–	4.8–5.8	5.4	5.3
Pol	% juice	>14	19.43	19.26
Purity	% juice	>85	92.44	92.42

Single batch results (n = 1).

TABLE 7 | Physicochemical parameters of example raw sugar lots produced from control cultivar CTC20 and CTC175-A including relevant copersucar raw sugar classification specifications.

Characteristic	Unit	CTC20	CTC175-A	Type 3C
Starch	mg/kg	294	207	–
Conductometric ash	% m/m	0.02	0.03	max 0.1
ICUMSA color (MOPS)	IU	200	246	max 400
Dextran	mg/kg	<10	<10	–
Filterability	mL–min	45–5	43–5	–
Acid beverage floc	–	Negative	Negative	–
Alcohol floc	Abs	0.055	0.083	–
RS	% m/m	<0.06	<0.06	–
Polarization	Z	99.74	99.67	min 99.5
Turbidity	NTU	15	40	–

Single batch results (n = 1).

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Detection of *cry1Ab* and *nptII* DNA Sequences of Fractions of Laboratory Processing of CTC175-A

The results of detection of gene sequences of DNA (*cry1Ab*, *nptII*, and endogenous *ubi*) in samples from the laboratory fractions of sugarcane processing showed the presence of endogenous DNA (*ubi* gene) in leaf, bagasse, primary juice, and in the precipitated fraction of clarification process filter cake, both from the laboratory processing of event CTC175-A and CTC20 (**Tables 8** and **9**). The molasses fractions from CTC20 but not from CTC175-A event also showed the presence of the *ubi* gene (**Tables 8** and **9**). The vinasse sample showed detection of *ubi* gene at levels equivalent to presence of a DNA concentration below 0.05 ng (positive control) (**Table 8**) and for the juice processed from CTC175-A (**Table 9**). Flegma also showed detection of *ubi* gene at levels equivalent to presence of a DNA concentration below 0.05 ng (positive control) (**Table 9**).

The results for amplification of *cry1Ab* gene from CTC175-A event were completely concordant with the results of *ubi* amplification in CTC175-A and CTC20, revealing positive amplifications for the less processed fractions (leaf, bagasse, and primary juice) and the precipitated residue filter cake (**Table 8**). Additionally, vinasse from CTC175-A also showed detection of *cry1Ab* gene at levels equivalent to presence of a DNA concentration below 0.05 ng, as for *ubi* gene from CTC175-A and CTC20. All samples spiked with 0.05 ng of DNA from *cry1Ab* gene showed expected amplifications, ensuring the absence of matrix negative influence on DNA amplifications, at least at the level of quantification of this assay. These results clearly show that the *cry1Ab* DNA was degraded and/or removed in the juice clarification step, and subsequent downstream fractions, including raw sugar, did not contain detectable levels of *cry1Ab* gene DNA.

The results of detection of *nptII* gene are similar to those for *cry1Ab* detection (**Table 9**). There was DNA detection in all processing samples spiked with appropriated amount to detect 0.05 ng of *nptII* DNA (positive controls) showing that the gene, if present could be amplified and detected in each fraction. Samples obtained from byproducts of CTC20 processing detected DNA presence only for the *ubi* gene in unprocessed samples (leaves) or less processed (bagasse, juice, and filter cake), except for molasses which also showed amplification. The flegma sample showed detection equivalent to a DNA concentration below 0.05 ng (**Table 9**). Samples from of event CTC175-A event showed DNA presence for both the genes (*ubi* and *nptII*) in samples without processing (leaf) or minimal processing (bagasse, juice, and filter cake) as described for detecting the endogenous gene in the CTC20 cultivar (**Table 9**).

These results obtained by TaqMan assay are consistent with the findings of Cullis et al. (2014) who also found a dramatic reduction in total sugarcane DNA quantity upon production of the clarified juice, the common starting material for production of raw sugar and ethanol production. No heterologous DNA was detected by PCR amplification in the final products raw sugar or flegma (the starting material for ethanol production).

Detection of Total Proteins in Fractions of Laboratory Production of Sugar and Ethanol

Protein quantification methodology was effective in detecting measurable amounts of protein (above LOD) for samples of leaves, bagasse, and primary juice from event CTC175-A and the CTC20 conventional (**Table 10**) collected in the laboratory-scale preparation of sugar and ethanol. The values presented at **Table 10** should be evaluated as single measures and not as estimates of total protein content in fractions of processing of CTC175-A and CTC20 because there was only one preparation for each material. It was not possible to detect measurable total proteins in samples after juice clarification for both, CTC175-A

TABLE 8 | Results of gene amplification of *ubi* (endogenous gene) and *cry1Ab* in samples collected during the laboratory sugarcane processing to produce sugar and ethanol from CTC20 and CTC175-A.

	CTC20 + 0.05 ng DNA (*cry1ab*)		CTC20		CTC175-A	
	ubi	*cry1ab*	*ubi*	*cry1ab*	*ubi*	*cry1ab*
Leaf	+	+	+	–	+	+
Bagasse	+	+	+	–	+	+
Primary juice	+	+	+	–	+	+
Filter cake	+	+	+	–	+	+
Clarified juice	+	+	–	–	–	–
Syrup	+	+	–	–	–	–
Molasses	+	+	+	–	–	–
Sugar	+	+	–	–	–	–
Flegma	+	+	–	–	–	–
Vinasse	+	+	<LOD	–	<LOD	<LOD
Evaporation water	+	+	–	–	–	–
Condensation water	+	+	–	–	–	–

Samples obtained from cultivar CTC20 were intentionally spiked with 0.5 and 0.05 ng of DNA from CTC175-A event. +: presence; –: absence; <LOD: the amplification curve shows cq later than the same sample at a concentration of 0.05 ng of total DNA per reaction.

TABLE 9 | Results of gene amplification of *ubi* (endogenous gene) and *nptII* in samples collected during the sugarcane laboratory processing to produce sugar and ethanol from CTC20 and CTC175-A.

	CTC20 + 0.05 ng DNA (*nptII*)		CTC20		CTC175-A	
	ubi	*nptII*	*ubi*	*nptII*	*ubi*	*nptII*
Leaf	+	+	+	–	+	+
Bagasse	+	+	+	–	+	+
Primary juice	+	+	+	–	+	+
Filter cake	+	+	+	–	+	+
Clarified juice	+	+	–	–	<LOD	<LOD
Syrup	+	+	–	–	–	–
Molasses	+	+	+	–	–	–
Sugar	+	+	–	–	–	–
Flegma	+	+	< LOD	–	<LOD	–
Vinasse	+	+	–	–	+	–
Evaporation water	+	+	–	–	–	–
Condensation water	+	+	–	–	–	–

Samples obtained from cultivar CTC20 were intentionally spiked with 0.5 and 0.05 ng of DNA from CTC175-A event. +: presence; –: absence; <LOD: the amplification curve shows cq later than the same sample at a concentration of 0.05 ng of total DNA per reaction.

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TABLE 10 | Total protein quantification after Bradford extraction in fractions of laboratory sugarcane processing.

Sample	Total protein (mg/mL)	
	CTC175-A	CTC20
Leaf	0.64	0.79
Bagasse	0.05	0.02
Primary juice	0.06	0.12
Clarified juice	Not detected	Not detected
Filter cake	Not detected	Not detected
Syrup	Not detected	Not detected
Molasses	Not detected	Not detected
Sugar	Not detected	Not detected
Phlegma	Not detected	Not detected
Vinasse	Not detected	Not detected
Evaporation water	Not detected	Not detected
Condensation water	Not detected	Not detected

Single batch results (n = 1).

and CTC20, indicating this process leads to either protein degradation or precipitation.

Detection of Cry1Ab in Raw Sugar Produced in Laboratory Scale

Cry1Ab protein was detected in leaves, bagasse, and primary juice produced in laboratory from event CTC175-A sugarcane. The remaining samples that have undergone various chemical and/or heat treatments during the manufacturing process of obtaining sugar and alcohol, as well as the final processed products (sugar and flegma) showed no detectable Cry1Ab protein (**Table 11**). As expected, samples from CTC20 cultivar did not contain detectable Cry1Ab protein whereas samples from CTC20 spiked with Cry1Ab protein showed detection of protein in all cases, demonstrating lack of matrix interference with the detection assay. Molasses and vinasse samples were reported as <LOD because, although there was antibody reaction for these samples, the OD reading was below the lowest point of the dilution curve (3.125 ng).

Detection of DNA, Total Proteins, and RuBisCo in Fractions of Industrial Processing

The SDS-PAGE evaluation from samples from diffuser mill revealed a protein smear in all samples (Figure S1A in Supplementary Material). It was possible to detect smeared proteins in samples of leaves, primary juice and filter cake. It was not possible to detect total proteins from clarified juice and downstream samples (syrup, molasses, and raw sugar) (Figure S1A in Supplementary Material). It was not possible to detect proteins in raw sugar samples (Figure S1B in Supplementary Material) produced in tandem roller and diffuser mills.

The evaluation of total DNA from samples from the Brazilian mill processing fractions revealed that the quantity of extracted total DNA ranged from 173 ng/μL in bagasse to 1.36 ng/μL in clarified juice from samples derived from the tandem roller mill (**Table 12**). Raw sugar and flegma were below the LOD of Qubit® Quantitation Assay Kit (0.2 ng). Samples from the diffuser mill presented as much as 16.6 ng of DNA per μL of bagasse to approximately 1 ng/μL in the molasses fraction. Samples from raw sugar, flegma, clarified juice, and syrup were below of LOD of Qubit® reagents (**Table 12**). Samples from sugarcane leaves yielded as much as 880 ng/μL of DNA.

TABLE 11 | Presence (+) and absence (–) of Cry1ab protein in fractions samples from the industrial processing of CTC20 and CTC 175-A Varieties, using ELISA methodology.

Samples	CTC175-A	CTC20	CTC20 + Cry1ab
Leaf	+	–	+
Bagasse	+	–	+
Primary juice	+	–	+
Clarified juice	–	–	+
Filter cake	–	–	+
Syrup	–	–	+
Molasses	–	–	<LOD
Sugar	–	–	+
Phlegma	–	–	+
Vinasse	–	–	<LOD
Evaporation water	–	–	+
Condensation water	–	–	+

<LOD: below protein detection level per the dilution curve of known concentrations.

TABLE 12 | DNA quantification for each processing fraction sample from two types of mills.

DNA quantification		
Sample	**Tandem roller mill (ng/μL)**	**Diffuser mill (ng/μL)**
Leaves CTC20	880.0	
Bagasse	173	16.6
Primary juice	11.5	6.4
Clarified juice	1.36	<LOD
Filter cake	26.8	16.5
Syrup	1.84	<LOD
Molasses	2.69	0.93
Vinasse	37.2	26.8
Raw sugar	<LOD	<LOD
Flegma	<LOD	<LOD

Single batch results (n = 1).

TABLE 13 | RuBisCO DNA detection in fractions collected during industrial processing of sugar and ethanol production from two types of sugarcane mills.

	Diffuser mill		Tandem roller mill	
	Fraction samples	Fraction samples spiked (+)	Fraction samples	Fraction samples spiked (+)
Bagasse	+	+	+	+
Primary juice	+	+	+	+
Filter cake	+	+	+	+
Clarified juice	<LOD	[a]	+	+
Raw sugar	<LOD	[a]	<LOD	[a]
Flegma	<LOD	[a]	<LOD	[a]
Vinasse	+	[b]	+	[b]
Leaf	+		+	

+: positive detection; <LOD: below of limit of detection; spiked (+): samples contaminated with DNA detection of 0.01 ng (nanogram).

[a]Positive amplification for samples contaminated with DNA 3 pg of DNA.

[b]Positive amplification for samples contaminated with DNA 100 pg of DNA.

The values presented at **Table 12** should be evaluated as single point estimates of DNA content in fractions from tandem roller and diffuser Brazilian mills. Overall, samples from both mills showed a trend of decreasing DNA concentration throughout the processing steps. The final products of processing (raw sugar and flegma) did not presented DNA above the LOD of this assay for both types of mills (**Table 12**).

Primer combinations 1 and 2 (**Table 3**) were used to obtain results of RuBisCo DNA detection with samples from both types of mills. The results from diffuser mill showed RuBisCo DNA detection for all samples except clarified juice, raw sugar, and flegma. The positive controls (i.e., spiked samples) yielded amplification down to the 0.01 ng level for all samples except vinasse, which could only be observed at the 0.1 ng level. Spiked samples of clarified sugar, raw sugar and flegma supported amplification at 3 pg of DNA. The results from tandem roller mill fractions were similar with those found from diffuser mill samples and indicates that RuBisCo DNA can be detected in all processing fractions except raw sugar and flegma (**Table 13**). The positive controls (i.e., spiked samples) yielded amplification down to the 0.01 ng level for all samples except vinasse, which could only be observed at the 0.1 ng level. Spiked samples of raw sugar and flegma supported amplification at 3 pg of DNA. Therefore, these results are concordant in not identifying RuBisCo DNA in raw sugar and flegma.

TABLE 14 | Total protein quantification using BCA in fraction collected from two types of mills.

Sample	Diffuser mill total protein (µg/mL)	Tandem roller mill total protein (µg/mL)
Leaf	~1,500	
Bagasse	133	267
Primary juice	1,319	1,237
Filter cake	623	933
Clarified juice	13	54
Syrup	8	24
Molasse	88	418
Raw sugar	4	10
Flegma	1	9
Vinasse	581	284

Single batch results (n = 1).

The values obtained for Total protein and RuBisCo quantification in processing fractions of tandem roller and diffuser Brazilian mills should be evaluated as single point estimates. In samples collected from mills, BCA protein quantification demonstrated that most of protein content present at sugarcane juice is eliminated in the precipitated filter cake (**Table 14**) resulting in a protein content in clarified juice at least two orders of magnitude lower than in primary juice. The final protein content in raw sugar and in flegma is minimal (**Table 14**).

Results of RuBisCo quantification were concordant with evaluation of BCA total protein quantification. **Figure 3** shows the results for ELISA assay for RuBisCO concentrations in samples

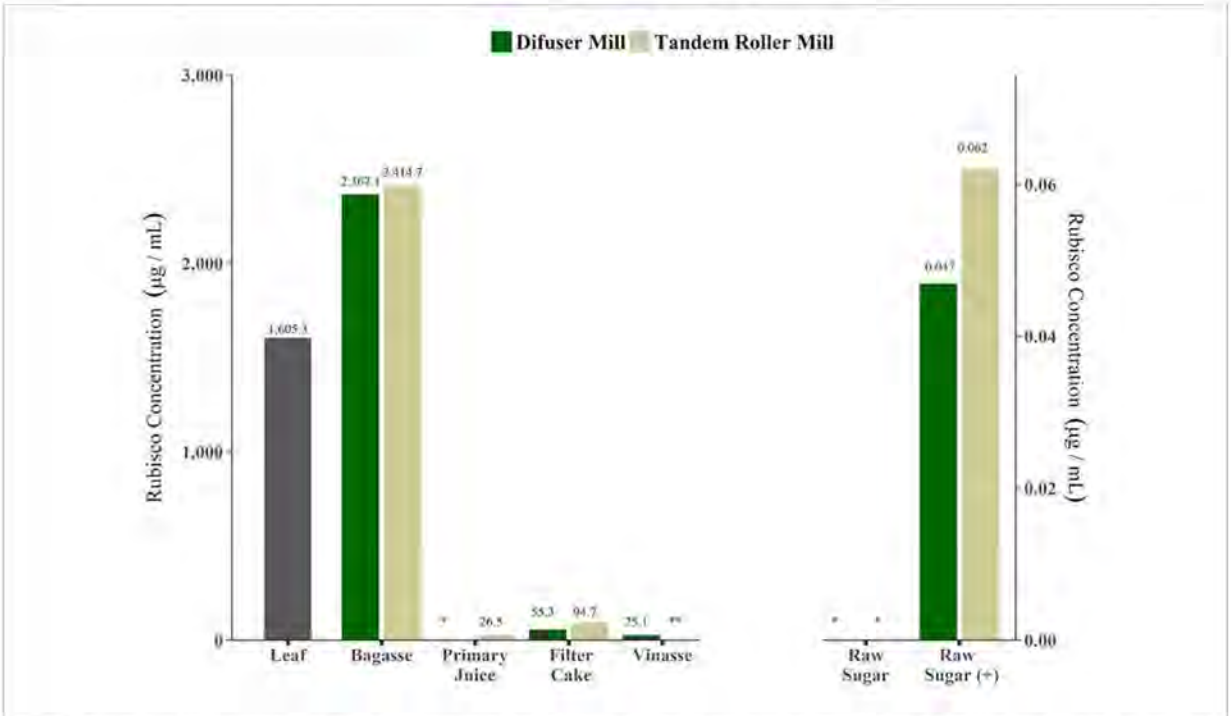

FIGURE 3 | Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) protein detection in by product samples collected during industrial process for sugar and alcohol production, from two types of sugarcane mills, by ELISA assay. Single batch results ($n = 1$). *: below limit of detection (LOD). **: not analyzed. Raw sugar (+): samples of raw sugar spiked with 10 µg of total protein before extraction.

from fractions obtained from commercial tandem and diffuser mills processing plants. Leaf and bagasse are far above the range of quantification (3.12–800 µg/mL), therefore are not accurate estimates. RuBisCO protein was also detected in samples of primary juice, filter cake and vinasse. In the case of primary juice, it was not possible to quantify RuBisCO in samples from diffuser mill. Samples of vinasse from tandem roller mill were not analyzed. As expected, the concentrations of RuBisCO were below the LOQ in samples of raw sugar produced from both type of mills. In samples of raw sugar spiked with 10 µg of RuBisCO protein before extraction, ELISA was sensitive enough to detect as little as 0.05 or 0.06 µg/mL of RuBisCO, confirming that the protein could have been detected at those levels if it was present in raw sugar.

CONCLUSION

The results presented in this study demonstrates that event CTC175-A presents very low expression of Cry1Ab and nptII proteins in stalks, the raw material for sugar and ethanol production. This result is in agreement with the design of the DNA cassette used to obtain this event, that was constructed to drive high levels of Cry1Ab in leaves. Besides, several assays of fractions of laboratory processing strongly suggests that total DNA, total protein, heterologous DNA and Cry1Ab protein are degraded during processing, leading to concentrations that are not easily detected by commonly used methodology employed to evaluate the presence of GMOs or GMOs derivative in food/feed.

Three lines of evidence clearly establish that raw sugar does not contain detectable levels of either the inserted heterologous DNA or expressed proteins. First, published studies of total DNA and protein loss during stalk processing to refined sugar showed levels of <1 pg total DNA/g refined sugar and ~1 µg total protein/g refined sugar (Cullis et al., 2014). Given these extremely low detection levels for total DNA and protein, it is expected that the small quantities of heterologous DNA and newly expressed protein would also be no detectable. Second, studies presented herein (**Tables 11–14**; **Figure 3**), tracked the concentrations of total protein and RuBisCO protein during stalk processing to refined sugar in two types of commercial processing plants in Brazil. RuBisCO is the single most abundant stalk protein (up to 30% of total plant protein) with very high DNA copy number. These studies confirmed the results of Cullis et al. (2014) that the extent of protein loss during processing is at least three to four orders of magnitude (2–5 mg of total protein per gram of cane preextraction and 0.75–1.875 µg total protein in raw sugar derived from 1 g of cane). Finally, and most importantly, studies with new event CTC175-A sugarcane stalks, clarified juice, molasses, and raw sugar showed no detectable levels of Cry1Ab protein (by ELISA, <235 ng/g FW tissue) in stalks or processed fractions. Similarly, no heterologous DNA was detected in clarified juice and downstream products including raw sugar. These results are in agreement with the results of other studies that investigated the degradation of specific DNA fragments inserted into genetically modified sugarcane (NptII) or glyphosate-resistant sugar beet (CP4 EPSPS) that reported the complete elimination of the inserted DNA during processing to refined sugar (Klein et al., 1998; Oguchi et al., 2009; Joyce et al., 2013).

In conclusion, results reported here demonstrate lack of detectable protein and DNA from CTC175-A at reasonable levels of sensitivity in processing fractions of sugarcane, including raw sugar, and are in alignment with previous studies reported in Cullis et al. (2014) and Joyce et al. (2013) on sugarcane. Detectability and quantification of these analytes (proteins in particular) are directly relevant to the globally accepted comprehensive safety assessment strategy on biotechnology-derived crops. Quantification forms the underpinning for the exposure component of the risked-based safety assessment; low/no exposure to the heterologous proteins expressed in CTC175-A in conjunction with the extensively reviewed hazard assessment data on those proteins showing no measurable toxicity to humans, animals, or the environment, support the safety conclusions on CTC175-A. Currently, there are no regulations specific to sugarcane related to DNA or protein detection; this work seeks to establish viable parameters to determine levels of exposure to potential toxicants. It is though publications such as this that government and industry standards can be derived and justified.

ETHICS STATEMENT

All manipulation of genetically modified organisms and their derivatives were strictly performed according to Brazilian Biosafety Law 11.105 and CTNBio regulations and required approvals.

AUTHOR CONTRIBUTIONS

ACG, RL, and WO conceived the study; AG, GM, MS, and TF performed experiments; ACG, RL, AG, and MS analyzed the data; ACG, AG, GM, RL, and WO wrote the manuscript; DO, GM, and MS provided supportive information.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fbioe.2018.00024/full#supplementary-material.

FIGURE S1 | SDS-PAGE gel of protein in fractions of Brazilian mills. M, molecular 838 Marker; BG, bagasse; PJ, primary Juice; FC, Filter Cake; CJ, Clarified juice; SY, syrup; MO, 839 molasses; X, empty lane, L, leaf; RW, Raw sugar. Number following letters indicates spiking of 840 correspondent amount (in µg) of total total protein before protein extraction.

TABLE S1 | Supplemental methodology.

REFERENCES

Aljanabi, S. M., Forget, L., and Dookun, A. (1999). An improved and rapid protocol for the isolation of polysaccharide- and polyphenol-free sugarcane DNA. *Plant Mol. Biol. Rep.* 17, 1–8. doi:10.1023/A:1007692929505

ASBC. (1976). *Methods of Analysis, Method Beer 26*. St. Paul, MN, USA: American Society of Brewing Chemists. Available at: http://methods.asbcnet.org/summaries/beer-26.aspx (accessed August 11, 2016).

Botelho, P. S. M., and Macedo, N. (2002). "Cotesia flavipes para o controle de *Diatraea saccharalis*," in *Controle biológico no Brasil: Parasitoides e predadores*, eds J. R. P. Parra, P. S. M. Botelho, B. S. Correaferreira, and J. M. S. Bento (São Paulo: Manole), 409–425.

Brokensha, M. A. (1998). "Chapter 5 – reception, storage and washing," in *Sugar Technology Beet and Cane Sugar Manufacture*, eds P. W. van der Poel, H. Schiweck, and T. Schwartz (Berlin: Verlag Dr. Albert Vartens KG).

Bruijn, J. (1998). "Chapter 12 – crystallization," in *Sugar Technology Beet and Cane Sugar Manufacture*, eds P. W. van der Poel, H. Schiweck, and T. Schwartz (Berlin: Verlag Dr. Albert Vartens KG).

Cheavegatti-Gianotto, A., de Abreu, H. M., Arruda, P., Bespalhok Filho, J. C., Burnquist, W. L., Creste, S., et al. (2011). Sugarcane (Saccharum X officinarum): a reference study for the regulation of genetically modified cultivars in Brazil. *Trop. Plant Biol.* 4, 62–89. doi:10.1007/s12042-011-9068-3

Christensen, H. A., Sharrock, A. R., and Quail, H. (1992). Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* 18, 675–689. doi:10.1007/BF00020010

Cullis, C., Contento, A. L., and Schell, M. A. (2014). DNA and protein analysis throughout the industrial process of refining sugar cane. *Int. J. Agric. Food Res.* 3, 1–15.

Damaj, M. B., Kumpatla, S. P., Emani, C., Beremand, P. D., Reddy, A. S., Rathore, K. S., et al. (2010). Sugarcane DIRIGENT and O-methyltransferase promoters confer stem-regulated gene expression in diverse monocots. *Planta* 231, 1439–1458. doi:10.1007/s00425-010-1138-5

Fraley, R. T., Rogers, S. G., Horsch, R. B., Sanders, P. R., Flick, J. S., Adams, S. P., et al. (1983). Expression of bacterial genes in plant cells. *Proc. Natl. Acad. Sci. U.S.A* 80, 4803–4807. doi:10.1073/pnas.80.15.4803

Garcia, W. T. (2012). *Curso gerencial de tecnologia sucroenergética – Controle de Qualidade*.

Harrison, M. D., Geijskes, J., Coleman, H. D., Shand, K., Kinkema, M., and Palupe, A. (2011). Accumulation of recombinant cellobiohydrolase and endoglucanase in the leaves of mature transgenic sugar cane. *J. Plant Biotechnol.* 9, 884–896. doi:10.1111/j.1467-7652.2011.00597.x

Hugot, E. (1969). *Manual da engenharia açucareira*, Vol. II. São Paulo: Mestre Jou.

ICUMSA – Methods Book. Method GS 1/2/3/9-1. (2011a). *The Determination of the Polarisation of Raw Sugar by Polarimetry – Official*. Berlin, Germany: Verlag Dr. Albert Bartens KG. Avaliable at: http://www.icumsa.org/index.php?id=120

ICUMSA – Methods Book - Method GS 1/2/9-15. (2011b). *The Determination of Dextran in Sugars by a Modified Alcohol Haze Method*. Berlin, Germany: Verlag Dr. Albert Bartens KG. Avaliable at: http://www.icumsa.org/index.php?id=120

ICUMSA – Methods Book – Method GS 9/1/2/3-8. (2011c). *The Determination of Sugar Solution Colour at pH 7.0 by the MOPS Buffer Method Official*. Berlin, Germany: Verlag Dr. Albert Bartens KG. Avaliable at: http://www.icumsa.org/index.php?id=120

ICUMSA – Methods Book - Method GS 1-16. (2009). *The Determination of Starch in Raw Sugar by a Modified the BSES Method*. Berlin, Germany: Verlag Dr. Albert Bartens KG. Avaliable at: http://www.icumsa.org/index.php?id=120

ICUMSA – Método GS 1/3/4/7/8-13. (1994). *The Determination of Conductivity Ash in Raw Sugar, Grown Sugar, Juice, Syrup, and Molasses – Official*. Berlin, Germany: Verlag Dr. Albert Bartens KG. Avaliable at: http://www.icumsa.org/index.php?id=120

Joyce, P. A., Dinh, S-Q., Burns, E. M., and O'Shea, M. G. (2013). Sugar from genetically modified sugarcane: tracking transgenes, transgene products and compositional analysis. *Proc. Int. Soc. Sugar Cane Technol.* 28, 1–9.

Klein, J., Altenbuchner, J., and Mattes, R. (1998). Nucleic acid and protein elimination during the sugar manufacturing process of conventional and transgenic sugar beets. *J. Biotechnol.* 60, 145–153. doi:10.1016/S0168-1656(98)00006-6

Koziel, M. G., Desai, N. M., Lewis, K. S., Kramer, U., Warren, G. W., Evolva, S. V., et al. (1992). *Method to Produce a Sequence Coding for Protein Corn, Optimized Process for Corn Plants Protection and Method for Production of a Protein Insecticidal Bacillus thuringiensis (BT)*.

Merheb, G. A. (2014). *Influência da contaminação combinada de dextrana e amido na cristalização do açúcar*. PhD Thesis, UFSCar, São Carlos. Available at: https://repositorio.ufscar.br/handle/ufscar/3961 (accessed August 11, 2016).

Merheb, G. A., Oliveira, N., Giulietti, M., and Bernardo, A. (2016). Combined effect of starch and dextran in sucrose crystallization. *Sugar Industry* 141, 697–704.

Novello, A. P. (2015). *Seleção de leveduras para fermentação com alta pressão osmótica usando processo de fermentação extrativa*. Piracicaba: Esalq. PhD Thesis, Esalq, Piracicaba. doi:10.11606/T.11.2015.tde-29042015-102100

OECD. (2011). "Consensus document on compositional considerations for new varieties of sugarcane (Saccharum ssp. hybrids): key food and feed nutrients, anti-nutrients and toxicants," in *Series on the Safety of Novel Foods and Feeds No. 23* (Paris: OECD Environment Dictorate). Available at: https://www.oecd.org/env/ehs/biotrack/48962816.pdf (accessed March 2018).

Oguchi, T., Onishi, M., Chikagawa, Y., Kodama, T., Suzuki, E., Kasahara, M., et al. (2009). Investigation of DNA in residual sugar from sugar beet (*Beta vulgaris* l.). *Shokuhin Eiseigaku Zasshi* 50, 41–46. doi:10.3358/shokueishi.50.41

Oliveira, A. D., Spoto, M. H. F., Canniatti-Brazaca, S. G., Sousa, C. D., and Gallo, C. R. (2007a). Efeitos do processamento térmico e da radiação gama na conservacção de caldo de cana puro e adicionado de suco de frutas. *Ciência e Tecnologia de Alimentos* 27, 863–873. doi:10.1590/S0101-20612007000400029

Oliveira, D. T., Esquiaveto, M. M. M., and Silva Junior, J. F. (2007b). Impacto dos itens da especificação do açúcar na indústria alimentícia. *Ciência e Tecnologia de Alimentos* 27, 99–102. doi:10.1590/S0101-20612007000500018

Precetti, A. A. C. M., and Téran, F. O. (1983). "Gorgulhos da cana-de-açúcar, Sphenophorus levis Vaurie, 1978, e Metamasius hemipterus (L., 1765) (Col., Curculionidae)," in *Reunião Técnica Agronômica: Pragas da Cana-de-Açúcar*, Vol. 1 (Anais, Piracicaba: Copersucar), 32–37.

Precetti, A. A. C. M., Téran, F. O., and Sanchez, A. G. (1988). Alterações nas características tecnológicas de algumas variedades de cana-de-açúcar, devidas ao dano da broca Diatraea saccharalis. *Boletim Técnico Copersucar* 40, 3–8.

Santos, F., and Borém, A. (2013). *Cana-de-açúcar: do plantio à colheita*. Viçosa: UFV, 257.

Santos, F., Borém, A., and Caldas, C. (2012). *Cana-de-açúcar, Bioenergia, Açúcar e Etanol, Tecnologias e Perspectivas*, 2nd Edn. Viçosa: UFV, 627.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. (2006). *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples*. Golden: National Renewable Energy Laboratory, 11.

The Laboratory Manual for Australian Sugar Mills. (2001). *Method 32 – Reducing Sugars – Determination in Raw Sugar by the Lane and Eynon Method*. Available from: https://sugarresearch.com.au/wp-content/uploads/2017/02/Milling-laboratory-manual.pdf

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